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ENVIRONMENTAL STRESSES AND SKELETAL DEFORMITIES IN
FISH
FROM THE WILLAMETTE RIVER
OREGON, USA

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Final Report:

Environmental Stresses and Skeletal Deformities in Fish from the
Willamette River, Oregon, USA

By

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To: Oregon Watershed Enhancement Board

The Willamette River, one of only 14 American Heritage Rivers, flows through the most densely populated and agriculturally productive region of Oregon. Previous biological monitoring of Willamette River fish detected elevated frequencies of skeletal deformities in fish from certain areas of the lower (NP [NP], rivermile [RM] 26-55) and middle (near Wheatland Ferry [WF], RM 72-74) Willamette River, relative to those in the upper Willamette (i.e. near Corvallis [CV], RM 125-138). The objective of this study was to determine the likely cause of skeletal deformities in populations of Willamette River fish. Characterization of deformity loads in Willamette River fish collected in 2002 and 2003 demonstrated that deformity loads remained 2-3 times greater at the NPPool (NP) and WF locations than those observed at the CV location. There were some differences in water quality parameters between the NP and CV sites, but they did not readily explain the difference in deformity loads. Concentrations of bioavailable metals were below detection limits ($\approx 1-5 \mu\text{g/L}$). Concentrations of bioavailable polychlorinated biphenyls (PCBs) and chlorinated pesticides were generally below 0.25 ng/L. Concentrations of bioavailable polycyclic aromatic hydrocarbons were generally less than 5 ng/L. Chlorpyrifos (averaged less than 1.5 ng/L) was the only organophosphate pesticide detected as bioavailable in water. Concentrations of most persistent organic pollutants were below detection limits in ovary/oocyte tissue samples and sediments and those that were detected were not significantly different among sites. Bioassay of Willamette River water extracts provided no evidence that unidentified compounds or the complex mixture of compounds present in the extracts induced skeletal deformities in cyprinid fish. However, metacercariae of a digenean trematode were directly associated with a large portion of the lesions detected in fish collected from the Willamette River and the lesions were reproduced in fathead minnows exposed to cercariae extracted from field collected snails. As a whole, there was very little evidence to suggest that chemical contaminants were responsible for the greater deformity loads observed at NP and WF. Instead, the weight of evidence suggests that parasitic infection was the primary cause of skeletal deformities observed in Willamette River fish.

The Willamette River in western Oregon is one of only 14 American Heritage Rivers (Uhrich and Wentz 1999). The river is the 13th largest river in the United States in terms of stream flow and yields more runoff per square mile than any other river in the U.S (Uhrich and Wentz 1999). It flows north from Eugene for approximately 187 miles through mixed agricultural and urban areas to Portland, Oregon's largest metropolitan area, before joining the Columbia River just 10 feet above sea level (Figure 1). The Willamette basin is home to 70% of Oregonians and the Willamette Valley is renowned as one of the most highly productive agricultural regions in the Pacific Northwest (Wentz et al. 1998, Altman et al. 1997). The Willamette River provides a significant migratory corridor, nursery habitat and adult forage for runs of salmon, and nearly 50 species of fish have been identified in the river (Altman et al. 1997). Recreational or sport fishing is extremely popular, and resident species are fished throughout the year. Numerous animal species utilize the Willamette River during various seasons.

As part of on-going efforts to manage and protect aquatic life in the Willamette River basin, the Oregon Department of Environmental Quality initiated investigations of skeletal deformities in fish from the Willamette River in the early 1990s. Biological monitoring was widely used as a means to evaluate the health of aquatic ecosystems and the potential impacts of antropogenic activities on those systems. It was suggested that skeletal deformities in fish served as useful bioindicators of pollution (Bengtsson 1979; Valentine et al. 1972; Lemly 1997), and evaluation of skeletal deformities in juvenile fish was used extensively to monitor the health of fish populations (Sloof 1982, Bauman and Hamilton 1984, Moore and Hixson 1977, Bengtsson 1991, Lindesjö and Thulin 1992). In 1992-1994 the incidence of skeletal deformities in northern pikeminnow (NPM) (*Ptychocheilus oregonensis*;) collected from the NP (NP) region, extending from river mile (RM) 55 to 26.5 (Figure 1), ranged from 22-74% (Ellis 2000, Ellis et al. 1997). Skeletal deformity rates in NPM were also elevated (21.7%) in the middle Willamette River (around RM 72, Wheatland Ferry; Figure 1). In contrast, the skeletal deformity rates in juvenile NPM collected from the upper Willamette River (RM 185-125) ranged from 1.6-5.3% (Ellis

2000, Ellis et al. 1997). The NM was not the only species impacted. Of 15 species collected from the NP region, and associated tributaries, in 2000, skeletal deformity rates exceeded 25% in 10 species (Markle et al. 2002). As a whole, biomonitoring of skeletal deformities in Willamette river fish suggested that fish from the NP region and middle Willamette River, had significantly greater deformity rates than fish from the upper Willamette River.

In the mid-late 1990s, proposals to tap the NP region of the Willamette River as a source of drinking water for urban expansion heightened public concern related to the reports of deformed fish (<http://www.hevanet.com/safewater/recentnewshome.htm>). In 1998, for example, 85% of people surveyed expressed "extreme" concern about the level of toxic chemicals in the river (Oregon Daily Emerald, Feb. 26, 1998). In response to these concerns the 71st Oregon Legislative Assembly Enacted Senate Bill 234 in 2001. The bill "placed a high priority on gathering specific data about and improving the scientific understanding of the extent and probable cause of fish deformities in the Willamette River" (Oregon Senate Bill 234). This study was undertaken in direct response to that legislation.

A wide variety of chemical, physical, and biological stressors have been associated with the occurrence of skeletal deformities in fish. A variety of chemicals are known to induce neuromuscular damage that can result in skeletal deformities. These include heavy metals such as lead (Holcombe et al. 1976; Hodson et al. 1980; Bengtsson and Larsson 1986) and numerous organophosphate pesticides (McCann and Jasper 1972). Chemicals can also cause skeletal deformities by impairing developmental processes and bone formation. Compounds such as 2,3,7,8-tetrachlorodibenzo-*p*-dioxin, polyaromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs), toxaphene, and cadmium have been reported to cause skeletal deformities through such mechanisms (Henry et al. 1997, Mehrle et al. 1982, Mayer et al. 1978, Olsson et al. 1999, Teraoka et al. 2002, Cheng et al. 2000). In addition to chemicals, skeletal deformities have been linked to water quality problems including low pH (Frojnar 1977, Beamish 1977), low dissolved oxygen (Garside 1959, Alderdice et al. 1957), and elevated temperatures (Gabriel 1944, Kwain 1974). Nutritional deficits, particularly ascorbic acid and tryptophan deficiencies, have been linked to skeletal deformities in fish (Halver et al. 1969, Kloppel and Post 1975). Inbreeding has been

shown to cause skeletal malformations including scoliosis, lordosis, curved neural spines, fused vertebrae, and compressed vertebrae (McCay and Gjerde 1986, Ponyton 1987, Prion 1978). Finally, numerous infectious biological agents including viruses, bacteria, and parasites have been reported to cause skeletal deformities (Kent et al. 1989, La Patra et al. 2001, Hedrick et al. 1998, Matthews et al. 2001). Although the association of fish skeletal deformities with a wide variety of stressors makes it a useful endpoint for biological monitoring, the observation of a high incidence of skeletal deformities, alone, has little diagnostic value.

This study attempted to identify or diagnose the cause(s) of skeletal deformities associated with fish collected from the middle and lower Willamette River, with particular emphasis on the NP region. Skeletal deformities associated with fish collected from the upper, middle, and lower Willamette River in 2002 and 2003 were characterized to determine whether recent conditions were similar to those reported previously and to further describe spatial and temporal patterns of deformity loads. *In situ* monitoring of river water quality coupled with *in situ* sampling and analysis of bioavailable organic contaminants and metals compared water quality and potential for direct exposure to known chemical contaminants at the NP and CV study sites and determine whether these factors likely caused of the deformities. Analysis and comparison of sediment samples and fish tissue from NP versus CV, evaluated potential trophic or maternal transfer of known persistent organic pollutants (POPs) as a potential cause. Bioassay of river water potential to produce skeletal deformities in embryo-larval fathead minnows (FHM) (*Pimephales promelas*) under controlled laboratory conditions evaluated the potential role of unknown chemicals or complex mixtures in causing the skeletal deformities observed in Willamette River fish. Finally, field collected fish were examined for parasites, and the association of parasitic infection with skeletal lesions was quantified. Together, these components provided a weight-of-evidence-based, empirical, approach that identified the likely cause of skeletal deformities observed in fish collected from the NP region of the Willamette River.

Fish Collection and Deformity Characterization

Larval and juvenile fish were sampled from May-October 2002 and May-August 2003. Fish were collected by beach seine, cast net, and dip net. The three primary sampling areas were NP (RM 47.5-53; lower Willamette), Wheatland Ferry (WF; RM 72-74; middle Willamette) and CV (RM 125-138; upper Willamette). Specimens were fixed immediately in the field by immersion in 10% buffered formalin. Seventeen different species were collected, with NPM, *Richardsonius balteatus* (reidside shiner), *Catostomus macrocheilus* (largescale sucker), *Mylocheilus caurinus* (peamouth), and *Acrocheilus alutaceus* (chiselmouth), representing the most commonly collected species (total sample sizes > 1000; Cunningham et al. 2004-submitted).

Specimens fixed for a minimum of two weeks were X-rayed in a Faxitron MX-20 cabinet X-ray machine using AGFA Structurix D4 DW ETE industrial radiography film. Film was developed using a Kodak X-OMAT Model M6B developer. Radiographs were inspected for deformities using a 10-15X ocular over a light table. Radiographs of approximately 15,700 fish were examined. Presence or absence of twelve different categories of skeletal deformities were scored (Cunningham et al. 2004-submitted). Analyses were based on the number of deformity categories present per individual (deformity load) (Markle et al. 2002). Based on random reevaluation of 550 fish, reader error was not significant (Cunningham et al. 2004-submitted). Additional details regarding fish collection and deformity characterization are reported elsewhere (Cunningham et al. 2004-submitted).

In situ water quality and bioavailable contaminants

Site Description and Sample Collection: The sampling sites facilitated investigation of seasonal and spatial bioavailable contaminant concentrations in the Willamette River at NP and at upriver sites (CV) (Figure 1). Two stations were designated at NP, one on the south side of the river (RM 47; N 45° 16.02', W 122° 54.59') and one a few miles downriver on the north side of the river (RM 44; N 45° 15.27', W

122° 53.58'). In addition, two stations were designated at CV (RM 35; [1] N 45° 29.13', W 122° 39.06'; [2] N 45° 27.37', W 122° 39.47'). The NP 1 (RM 47) sampling station was about 25-30 feet from the shoreline and the water depth was 27 feet. NP 2 sample station (RM44) was about 30 feet from the shore the water depth was 20 feet. The CV sites were about 15 feet from the shore and the water depth was 7-11 feet. Flow near the CV sites in May 2002 was *ca.* 9,000-10,000 ft³/sec. By late July the flows had decreased by a factor of 2 to *ca.* 4,500 ft³/sec. Willamette River flow was greater at the NP sites. Flow in May 2002 was about 17,000 to 20,000 ft³/sec. By late July the flows decreased by a factor of 3 to about 7,000 ft³/sec. The CV sites were shallow (2-10ft) and characterized by shallow gravel and sediment riprap. In the NP area the Willamette was much deeper (20-60 feet) and there were no shallow gravel beds near the study sites.

Field sampling was conducted from May to July in 2002 and 2003. Three sampling campaigns were completed per year, one each in approximately *ca.* May, June and July. Each sampling event was 21 days. All samples (*in situ* sampling devices and grab samples) were collected 1 ft from the river bottom. The sampling campaigns were designed to capture the river conditions during fish egg laying and early fry development. Nutrient and water quality parameters including: dissolved oxygen, conductivity, specific conductance, salinity, total dissolved solids, resistivity, temperature, pH, ORP (oxidation reduction potential), depth, ammonium/ammonia, nitrate, and turbidity; were collected on an hourly basis with a YSI 6920 Sonde (YSI, Yellow Springs, OH).

Dissolved, bioavailable organic contaminants and metals were collected by deploying passive sampling devices (PSD) and diffusion gradient thin films (DGT) in protective mesh cages. PSDs consisted of neutral lipid (i.e. triolein) enclosed in layflat polymeric tubing (Environmental Sampling Technologies, St. Joseph, MO; Huckins et al. 1990). Five individual PSD and DGTs were included in each cage. Each cage was suspended with "float-cable-cage-cable-anchor" arrangement that ensured that the cage would stay at the station and would stay suspended one foot from the river bottom. The five PSD were later composited for analysis. PSD were kept on ice in sealed airtight containers during transport to and from field sites. Complete PSD descriptions have been previously published (Huckins et al.

1993). The PSD were were gently cleaned of any sediment or algae after deployment at the site utilizing a tub filled with site water to minimize air exposure. No fouling impedance was employed in the calculations of estimated water concentration since algae growth on the devices was nil to minimal.

Analytical Procedure: PSD were extracted by hexane dialyses, in amber jars. Sample volumes were reduced using a TurboVap. The samples were then run through gel permeation chromatograph (GPC) and fractions containing organochlorine and PAH contaminants were collected. Appropriate fractions were determined by analyzing standards and fortified samples (Sethajintanin et al. 2004-in press). Appropriate fractions were analyzed using GC-ECD (organochlorine contaminants) and HPLC-DAD and fluorescence (PAH contaminants). All sample manipulations were either performed in brown amber or foil wrapped containers to minimize UV/Vis exposure.

The organochlorine contaminants fraction was separately concentrated to 0.5 ml and analyzed by gas chromatography with dual capillary columns (DB-XLB and DB-17ms, J&W Scientific Inc.) and dual ECD detectors (Ni^{63}) with an injection volume of 2 μL . DB-XLB and DB-17ms (each was 30 m x 0.23 mm ID x 0.25 μm film thickness) were used for quantification and confirmation. The GC-ECD was a Varian Star $\text{\textcircled{R}}$ Model 3600 operated with the 8200 autosampler with the splitless mode. Helium and nitrogen were the carrier gas and makeup gas, respectively. Both columns were temperature programmed as follows: initial column temperature was 100 $^{\circ}\text{C}$ with a 1- min hold, then increased from 100 to 130 $^{\circ}\text{C}$ at 10 $^{\circ}\text{C}/\text{min}$, and from 130 to 285 $^{\circ}\text{C}$ at 3 $^{\circ}\text{C}/\text{min}$. The final temperature was held for 4 min. Injector and detector were set at 250 $^{\circ}\text{C}$ and 350 $^{\circ}\text{C}$, respectively. Chromatographic data were integrated and calculated using Varian Star 4.0- $\text{\textcircled{R}}$ software. Quantification was accomplished using five to eight standards. Correlation coefficients for the standard calibration curves were >0.998 . Analytes were reported when detected on both columns and only samples containing residues exceeding the blanks were considered positive.

Organophosphate insecticides were extracted as above and also analyzed by gas chromatography with a DB-17 column with electron capture detection and a DB-XLB column with a thermionic specific detector for nitrogen and phosphorus.

The PAH contaminant fraction was separately concentrated to 1.0 mL. PAH detection and quantitation was performed on a Hewlett Packard 1100 HPLC with dual detection by fluorescence or diode array, both with multiple wavelengths. The fluorescence detector had an excitation wavelength at 230 nm and emission wavelengths at 360, 410, and 460 nm; diode array had detection signals at 254, 242, and 230 nm. Only three compounds, fluorene, acenaphthylene, and indeno(1,2,3-cd)pyrene, were detected by diode array, the rest were detected with the fluorescence detector. The column used was a Phenomenex Luna C18, with 3 μ particle size. The instrument was run with a constant flow rate of 0.75 mL/min and a timed gradient for the acetonitrile / water eluent system. The time program ran at 40% acetonitrile for 10 min., was gradually ramped up to 70% acetonitrile for 15 min., and then ramped up to 90% acetonitrile for 10 min.. The program was held at 90% acetonitrile for 3 min. and then returned to 40% and analyzed by HPLC with diode array detection and fluorescence detection.

After the DGT were retrieved and in the laboratory, the resin-gel was removed and immersed for 24 h in 0.6 mL of 1M trace metal grade nitric acid. An aliquot was removed and diluted to 40 mL with 18M Ω *cm water. The pH was adjusted to ca. 4.5 to 5.5 prior to analysis by anodic stripping voltammetry (ASV). All grab water samples were filtered thru a 0.45 μ m membrane filter prior to metal analyses by ASV. ASV (TraceDetect, Seattle, WA) quantified the metals reported. Reduction potentials were verified with standards for each metal tested.

Quality control (QC): Field, trip, and extraction blanks were used with each sampling campaign. The field blank PSD sample were opened and exposed to the atmosphere during deployment or recovery. These field blanks were processed and analyzed exactly as deployed PSD samplers. Field extraction blanks, were opened in the field and washed simulating the process of removing the light sediment or algae on the passive sampling devices. Samples containing residues exceeding the blanks were considered positive for residues. Transport blank values were multiplied by the water volume they would have been exposed to if left with the other PSDs. The CV site was designated as field duplicate. Field duplicates represented 30% of all samples collected. All QC sample types were included in each analytical batch of analysis. Laboratory QC samples included reagent blanks, fortified samples and

laboratory duplicates, each Q type represented 5-10 % of the total number of samples analyzed in any given batch. They were prepared and analyzed in the same fashion as the field samples. Standard curves were typically composed of ≥ 4 standard concentrations for all organic analyses and ≥ 3 for all inorganic analyses.

Data Analysis: The theory and mathematical models required for estimation of analyte water concentrations from the concentration in the PSD lipid have been previously described (Huckins et al. 1993). The following equation was used to calculate the dissolved (bioavailable) water concentration:

$$C_w = C_{\text{spmd}} V_{\text{spmd}} / R_s t$$

Where C_w is the concentration of analyte in water, C_{spmd} is the concentration in lipid (spmd), t is the exposure time in days, V_{spmd} is the volume of lipid/membrane, and R_s is the PSD sampling rate. Sampling rates (R_s) for a large series of organochlorine and PAH contaminants have been previously established.

The mass of the metal in the DGT resin gel (M) was determined from the ASV quantitation. The theory and mathematical models required for estimation of the analyte water concentrations from the concentration in the DGT have been previously described. The following equation was used to calculate the labile (bioavailable) water concentration:

$$M = C_e (V_{\text{HNO}_3} + V_{\text{gel}}) / f_e$$

where C_e was the concentration of metals in the 1M HNO_3 elution solution, V_{HNO_3} was the volume of HNO_3 added to the resin gel, V_{gel} was the volume of the resin gel, and f_e was the elution factor for each metal. The concentration of the metal measured by DGT (C_{DGT} = "bioavailable" water concentration) was determined from the following equation:

$$C_{\text{DGT}} = M \Delta g / (D t A)$$

where Δg was the thickness of the diffusive gel (0.8mm) plus the thickness of the filter membrane (0.13 mm), D was the diffusion coefficient of metal in the gel, t was deployment time and A was the exposure area ($A=3.14 \text{ cm}^2$).

The Mann-Whitney test was applied for statistical comparisons of site differences in bioavailable contaminants.

Analysis of POPs in Northern pikeminnow ovary tissue

The NPM (*Ptychocheilus oregonensis*) was the species chosen for analysis of maternal transfer of POPs. They are abundant in both regions of the Willamette, are relatively easy to collect, reach moderately large sizes, and have been heavily impacted in the NP region (Markle et al. 2002; Ellis 2000). Adult NPM were collected from NP (N 45° 16.007, W 122° 55.031) and CV (N 44° 28.250, W 123° 14.300) study areas (Figure 1) in May-June 2002 using a combination of hook and line and electrofishing. Wet weight of the fish collected for analysis of maternally transferred POPs ranged from 375-975 g. There was no significant difference in the mean wet weights of the fish collected from the two study sites. Field collected animals were transported to the laboratory on ice. Ovary tissue and associated oocytes were removed from gravid females, using clean, solvent rinsed, dissection tools, and placed into certified I-Chem[®] jars. Tissue samples were stored at -20°C until extracted.

Samples were shipped to GLP- (Good Laboratory Practices) certified analytical laboratories for quantification of a range of POPs. Twenty-one chlorinated pesticides were quantified by gas chromatography with electron capture detection (GC/ECD) according to EPA method 8081A (ODEQ Laboratory, Portland OR). Twenty-eight polychlorinated biphenyl (PCB) congeners were quantified by GC/ECD according to EPA method 8082 (ODEQ Laboratory, Portland, OR). Additionally, concentrations of seven polychlorinated dibenzo-*p*-dioxin (PCDD) congeners and 10 polychlorinated dibenzofurans (PCDFs) were quantified by high resolution GC/MS (Axys Analytical, British Columbia, Canada). Method detection limits (MDLs) for chlorinated pesticides and PCBs ranged from 2.5-3.3 $\mu\text{g/Kg}$ wet wt. MDLs for PCDDs and PCDFs ranged from 0.10-0.13 ng/Kg wet wt.

A total of 5 ovarian tissue/oocyte samples (each from a separate fish) per study area were analyzed. For the purposes of statistical analysis and plotting of figures, concentrations below the method reporting limit (MRL) or detection limit were assumed to be equal to one half of this limit. When the assumptions of parametric statistics were met, t-tests were used to test for differences among study sites. Kolmogorov-Smirnov's test was used in cases where parametric assumptions were not met.

Analysis of POPs in sediment

Grab samples of surficial sediment were collected from the NP and CV region of the Willamette River. In 2002, three samples were collected at NP location N 45°16.308, W 122°59.460, and three samples were collected at CV location N 44°31.567, W 127°15.384. In 2003, three samples were collected at NP location N 45°15.567, W 122°54.231 and three samples were collected at CV location N 44°32.887, W 123°15.432. Sediment samples were scooped directly into certified I-Chem[®] jars and transported to the laboratory on ice. Samples were stored at -20°C until shipped for analysis. Sediment samples were extracted and analyzed at the ODEQ laboratory, Portland, OR. Sediment extracts were analyzed for 22 chlorinated pesticides by GC/ECD (EPA method 8081A), 8 nitrogen/phosphorous pesticides by GC/NPD, and 29 PCB congeners by GC/ECD (EPA method 8082 A). MDLs for chlorinated pesticides and PCBs were around 0.33 µg/Kg wet wt. The MDL for nitrogen/phosphorous pesticides was 10 µg/kg wet wt.

Preparation of River Water Extracts for Bioassay

Water samples were collected from four study locations during the summer of 2003. The sampling sites included two NP locations (NP: N 45°15.567, W 122°59.142 and AI N 45°16.145, W 122°59.142), Wheatland Ferry (WF: N 45°05.447, W 123°02.655), and CV (N 44°32.887, W 123°15.432). On each sampling day, samples were collected from CV and one of the other three sampling locations. Grab samples were collected in 20 L stainless steel containers and three 20 L samples were collected at each site. Samples were typically collected at a depth of approximately 1 m

and containers were opened and sealed (all air removed) underwater. In all cases, collections were made at least 30 cm below the surface and at least 30 cm above the sediment. Sample extraction was completed within 96 h of sample collection.

Samples were extracted by drawing them with vacuum through through DVB-phobic and DVB-phillic solid phase extraction disks (Bakerbond Speedisk[®] 8072-06, 8068-06, J.T. Baker, Phillipsburg, NJ) arranged in series (5 independent stations of phobic and philic disks in series). Flow rates were 15-30 ml/min. Each of the five Speedisk[®] stations was used to extract 12 L. After extraction was completed, the disks were dried under vacuum, and stored in airtight containers at -20°C overnight. Each disk was eluted three times with 5 ml of methanol. Eluents were passed through a column of Na_2SO_4 to remove any water present. Eluents from both the phobic and phillic disks were pooled together for each sample site (~150 ml) then evaporated to 3 ml under a steady stream of N_2 gas using a Zymark Turbovap II. Resulting extracts were transferred to amber glass vials and stored at -80°C until used for bioassay.

Since the extraction procedure was designed to capture a broad spectrum of known and unknown contaminants (wide range of K_{ows}), it was not feasible to determine extraction efficiencies for all possible components. The basic solid phase extraction approach described above has been characterized for over 100 current use pesticides and persistent organic pollutants (Usenko and Simonich 2004, personal communication). As a quality assurance test for this study, chlorpyrifos was spiked into water samples at multiple stages of the extraction process including. The greatest losses occurred during the solid phase extraction step. However, greater than 70% of the chlorpyrifos added to either river water or deionized water was recovered in hexane extracts of the final MeOH eluent. This was consistent with recoveries demonstrated by other researchers (Usenko and Simonich, 2003 personal communication). Overall, the extracts prepared were considered representative of a broad mixture of chemical contaminants present in the river, but quantitatively accurate proportions of each component was not assured.

Larval FHM less than 24 h post-hatch were obtained from Chesapeake Cultures (Hayes, VA, USA). Larval FHM (24-48 h post-hatch) were randomly assigned to 400 ml beakers containing 100 ml of dechlorinated tapwater (dtw). Each beaker was stocked with n=30 larval FHM. Beakers were then randomly assigned to one of eight treatment groups. The treatment groups for the study were as follows: control (CON; 200 ml of dtw); solvent control (SC; 0.05% MeOH in dtw); 8X-, 4X-, and 1X-CV; 8X-, 4X-, and 1X-NP, AI, or WF. 8X, 4X, 1X represents a volume of the appropriate extract dissolved in 200 ml dtw to provide a concentration equivalent to 800%, 400%, 100%, respectively, of the river water concentration of the extract's constituents assuming 100% recoveries. Methanol was added to each of the 4X and 1X treatments such that the total MeOH concentration was equivalent to that of the 8X treatments and SC (0.05%). Fifty percent of the test solution was renewed daily by drawing the solution down to 100 ml and adding 100 ml of fresh test solution containing nominal concentrations of extract and/or solvent. The location of each beaker on the exposure bench was assigned randomly.

After 5 days of exposure, surviving fish were counted and transferred to 1 L plastic containers for grow out to ~ d. 25-30 post-hatch. During the grow-out period fish were maintained in dtw supplied from a flow-through system. Throughout both the exposure and grow-out periods, water temperatures were maintained at 24-26°C, photoperiod was 16 h light, 8 h dark and FHM were fed *Spirulina* (Algae Feast, Earthrise, Petaluma, CA, USA) twice daily and brine shrimp nauplii (GSL Brine Shrimp, Ogden, UT, USA) once daily. Water quality (dissolved oxygen, pH, ammonia, and nitrite) was monitored daily.

At the end of the grow-out period, fish from each container were transferred, live, to a 5 cm diameter plastic tube with fine mesh at one end (PVC insert). The entire batch of fish was immersed in a 0.2% calcein (Sigma C-0875; St. Louis, MO, USA) solution (pH 7.0). Fish were live calcein stained for 10 min, transferred to clean water for 10 min to destain, and then euthanized by immersion in a 200 mg/L solution of MS-222 (Finquel®, Argent, Redmond, WA, USA). Euthanized specimens were immediately examined by fluorescence microscopy using a Leica MZFL111 dissecting microscope (Bartles and Stout, Bellevue, WA, USA) equipped with a mercury lamp and fluorescein/green

fluorescence protein filter. Calcium staining allows for direct visualization of calcified skeletal structures (Du et al. 2001). Each specimen was examined for skeletal deformities including scoliosis, lordosis, fused vertebrae, compressed centra, extra or missing spines, etc. Screening of several hundred fish as part of assay development confirmed that all these types of deformities were detectable by this method. Vertebral development was also scored on a scale of 1-5 using a criteria defined for this study. Digital images of each fish and close-ups of deformities, if detected, were captured and archived using ImagePro Plus 4.5.1 (Media Cybernetics, Silver Springs, MD, USA). In some cases, examinations were spread over 2-3 d. Replicates examined each day were selected randomly.

Survival to the end of the exposure period (6 d post-hatch), survival to the examination (28-30 d post-hatch), and percent of surviving fish with a skeletal deformity, and was determined for each replicate. Developmental score distributions were determined for each treatment. One-way analysis of variance was used to test for differences in survival or incidence of deformities, among treatments. A non-parametric Kruskal-Wallis test on ranks was used to test treatment-related differences in developmental score distributions.

Parasite Characterization and Laboratory Infection Assays

Characterization of parasite association with vertebral lesions in fish collected from the Willamette River was based on examination of histological sections of formalin preserved fish, as well as whole mounts of trypsin-cleared, alcian blue and alizarin red S-stained fish (Kent et al. 2004). The methods and statistical analysis used for the parasite characterization were reported elsewhere (Kent et al. 2004).

For laboratory transmission studies, laboratory-reared FHM were obtained from Chesapeake Cultures, Hayes, VA. Fish were held in dechlorinated tap water (23-26°C) to ensure unexposed fish did not become infected. Fish were maintained in static water aquaria with biological filters. Fish were delivered at 3 – 7 day old, and were initially feed paramecium cultures until about 10-14 days old, then switched to a mixture of brine shrimp naupallii (GSL Brine Shrimp) and freeze-dried Spirulina algae

(Algae Feast). After about 3 wk fish were then feed TetraMin flake food (Tetra Sales, Blacksburg, VA).

Fluminicola virens snails were collected from the NP area of the Willamette River (Figure 1) from June – August 2003. Cercariae consistent with those described by Niemi and Macy (1974) were harvested by holding individual snails in isolation in 24 well tissue culture plates in 2 ml water. For transmission studies, larval FHM of varying age (Table 4) were exposed to known concentrations of cercariae or control water. Several exposures were conducted as initial trials with very young fish resulted in high mortality in exposed fish (Table 4).

Incidence of infection, vertebral deformities, and association of worms with deformities was determined by examination of whole, preserved fish that were cleared with trypsin and stained with alcian blue and alizarin-red S (Dingerkus and Uhler 1977; Potthoff 1984). Fish were collected at either 55 or 70 days post exposure. Cleared fish were placed in a Petri dish, covered with glycerin, and the entire fish was examined at 25 or 50 X. Fish were also evaluated by radiography as described by Markle et al. (2002).

RESULTS & DISCUSSION

Deformity Loads in Willamette River Fish

Lack of reliable information on normal background deformity rates in unstressed fish populations limits value of skeletal deformities in fish as a biomonitoring tool. One study in salmonids suggests 2-5% may be a normal background rate of skeletal deformities in wild populations (Gill and Fisk 1966). However, it is unclear how much this background rate may vary among different species, and among geographically distinct populations. Since, in most cases, background deformity rates in unstressed populations are unknown, biomonitoring approaches based on skeletal deformities rely on the detection of changes in deformity loads from year to year, or on comparisons between locations with similar habitat, climate, etc. In the case of Willamette River fish marked geographic disparity in the

frequency of skeletal deformities in fish that suggest stress on fish populations in the NP and Wheatland Ferry regions of the river.

Deformity characterization for 2002-2003 were consistent with studies from previous years that suggested that the incidence of skeletal deformities in Willamette River fish were elevated in the lower (NP) and middle (Wheatland Ferry) Willamette River relative to up-stream areas (CV) (Ellis 2000, Markle et al. 2002). Among the five species most commonly sampled, the percent frequency of deformities was generally 2-3 times greater at the NP and Wheatland Ferry locations than at CV (Table 1). The only exception to this was the large-scale sucker (*Catostomus macrocheilus*), which was notable as the only catostomid species of the five most commonly collected. The other four species were cyprinids. Among the cyprinid species, mean deformity loads for fish from Wheatland Ferry were significantly greater than those for fish from CV (Table 1). The same was true for fish from NP, although for peamouth the difference was not statistically significant (Table 1). There were no obvious geographic or habitat differences that explained the differences observed (Cunningham et al. 2004). Overall, biomonitoring of skeletal deformities in fish collected at different locations along the Willamette River in 2002-2003 suggested fish populations near NP and Wheatland Ferry were stressed relative to those near CV.

Water Quality Characterization

Ammonium, nitrate, pH, temperature, dissolved oxygen, oxidative reduction potential, and specific conductance data were collected hourly during all 21 d sampling events. At each individual station, some parameters showed strong temporal and spatial variation while others did not. As expected, the water temperature varied during each 24 h cycle. The diurnal temperature variation was larger at the CV sites (1-2° C), whereas the NP sites varied 1°C or less over a 24 hr cycle. Temperature increased throughout the sampling season. In May, temperatures were generally 12 ± 1 C in May and by the end of the sampling period were ca. 22 ± 2 C. The temperature patterns were the same for both seasons (2002 and 2003). The pH varied from 7.2 to 7.8 at the NP sites for both 2002 and 2003. The

CV sites had larger diurnal changes in pH from 7.2 to 8.8 during some 24 hr cycles, usually in late May and early June. These large diurnal pH changes were seen in both 2002 and 2003. At all sites there were small decreases in pH from the 2002 season to the 2003 season. The nighttime pHs were similar at all locations; however, there was a strong geographic difference in daytime pH. The CV sites were often 1+ pH units higher than the NP sites. A few studies have linked low pH conditions (less than 5.5) to the development of skeletal deformities in suckers (Frojnar 1977; Beamish 1972), but we are not aware of any reports that link pH 7.2-8.8 conditions to skeletal deformities in fish.

The oxidation reduction potential (ORP) probe tended to drift after about 7-10 days of deployment. Since all deployments were 21 days, data after 7 days was excluded from the analysis. The ORP was consistently lower at the NP sites as compared to the CV sites. During some sampling events the difference was small, <10% lower, while for other events the difference was as great as a factor of 2. There was no apparent difference during the season at the CV sites, and little difference between 2002 and 2003. There was some evidence that the ORP increased during the season at the NP sites and there was some difference between 2002 and 2003.

The ORP value is a direct reading of the activity of the oxidizing and reducing agents in the water as they correspond to oxidation-reduction reactions. In general, the CV waters were more oxidizing than the NP waters, (or the NP waters are more reducing than the CV site water). Thus, based solely on ORP, microbial growth may have been favored at the NP site.

Specific conductivity (SC) was very similar for all sites (CV and NP). There was a slight increase at all sites as the seasons progressed (May-July). The SC pattern was similar for both 2002 and 2003. The dissolved oxygen (DO) pattern was the same for all sites. A diurnal pattern was apparent, and a slight decrease in DO was seen from May through July in both 2002 and 2003. A seasonal increase in ammonia was measured at all sites. The concentration increased about a factor of 5, from 0.05 to 0.25mg/L. In general, the CV sites had higher ammonia than the NP sites. The nitrate probe was not robust, in 2002 the drift generally occurred within 24 hr of deployment, in 2003 the probe failed within a few hours after deployment. The limited data indicated that nitrate was low early in the year (May) and

increased through July. Although the data was limited, it appeared that the nitrate concentrations were higher at the NP sites compared to the CV sites. As a whole, water quality monitoring provided no compelling evidence to suggest that differences in nutrient concentrations, pH, temperature, DO, or specific conductivity were likely causes of the different deformity loads observed at NP versus CV, but further investigation of a possible link between ORP differences and susceptibility to infection may be warranted.

Bioavailable Contaminants

Polycyclic aromatic hydrocarbons (PAHs): Total bioavailable PAHs were low at all locations, for all sampling events, (generally <5 ng/L). Of the 16 PAHs measured, 13 PAHs were below detection limits (<0.1 ng/L) in 2002. In 2003, 10 PAHs were typically below detection limits. Three PAHs, phenanthrene, fluoranthene, and anthracene, were detected at sites in 2002 and 2003. At the CV sites phenanthrene and anthracene were equally abundant in 2002, while fluoranthene was somewhat less abundant (Table 2). Of the three PAH detected at the NP sites in 2002, phenanthrene was the most abundant (Table 2). In 2003, anthracene, and phenanthrene were the most abundant of the 16 individual bioavailable PAHs. In 2002, the CV sites consistently had higher total PAH (Σ PAH) concentrations compared to the NP study sites (Table 2). The average Σ PAH in 2002 was *ca.* 3.2 ng/L at CV, while the other sites had Σ PAH concentrations in 2002 of *ca.* \leq 2 ng/L. In 2003 this pattern was consistent (Table 3). The average CV sites (1&2) had Σ PAH of 3.9 and 2.7 ng/L respectively, while the 2003 average NP sites (1&2) had Σ PAH of \leq 2 ng/L. At the low concentrations detected at the two sites, PAHs were not considered to be likely contributors to the difference in deformity loads associated with the two areas.

Polychlorinated biphenyls (PCBs) PCBs analysis for this study was based on a congener-specific approach. However, concentrations of all congeners were quite low, so interpretation of results focused on total PCB concentrations. The total bioavailable PCB concentrations were generally very low, < 0.15 ng/L at all sites and many sites were below detection limits (0.03 ng/L) (Table 2). During 2002 and 2003, the total bioavailable PCBs were generally higher at the NP sites (Table 2). However,

since most of the data are below detection limits or near detection limits, it is difficult to draw any firm conclusions. As a whole, the PCB concentrations detected did not readily explain the difference in deformity loads or raise alarm.

Pesticides: The bioavailable Σ DDT concentrations were low (generally < 0.3 ng/L) at all sites in 2002 and 2003 (Table 2). On average the NP sites had the same or higher concentrations of Σ DDT than the CV sites (Table 2). The difference between sites was significant in 2003 ($p=0.041$) but not in 2002 ($p=0.18$). There was no significant difference between Σ DDT in 2002 and 2003 ($p=0.589$ NP; $p=0.310$ CV). In 2002, the DDT profile was dominated by p,p-DDD followed by DDE and DDT (Table 2). This profile would be expected from older deposits. However, in 2003, DDE dominated, followed by DDD and DDT (Table 2). DDD and DDE concentrations detected in 2002 and 2003 were not significantly different ($p=0.065-1.0$). Mean DDT concentrations at NP were not significantly different between 2002 and 2003 ($p=0.699$; Table 2), but mean bioavailable DDT detected at CV increased 3.5-fold ($p=0.004$; Table 2). The cause of the increased p,p'-DDT concentration was not clear, but the results suggest input from a newer source.

Dieldrin concentrations were slightly higher at the NP sites compared to the CV sites (Table 2; 2002 $p=0.015$; 2003 $p=0.004$). However, the bioavailable concentrations of dieldrin were very low, <0.15 ng/L and there was no significant difference between years (NP $p=0.699$; CV $p=0.589$; Table 2). The dieldrin concentrations detected are well below those generally thought to be toxic to fish (Mayer and Ellersiek 1986; Georgacakis and Khan 1971) and we are not aware of any reports linking ng/L concentrations of dieldrin to skeletal deformities.

Chlorpyrifos was the only organophosphate pesticide detected. Dimethoate, diazinon, and azinophos-methyl were below detection limits at all sites for all sampling campaigns (detection limits were estimated at 2, 3 and 2 ng/L, respectively). In 2003, the estimated, bioavailable, water concentration for chlorpyrifos averaged 0.74 ± 0.51 at CV, and 1.38 ± 0.33 ng/L at NP (Table 2). There was a trend for higher chlorpyrifos concentrations in NP but this difference was not statistically significant ($P=0.065$). The bioavailable chlorpyrifos concentrations observed in this study were well

below those reported to be toxic to fish (Giesy et al. 1999) and there were no peer-reviewed reports linking lesser concentrations of chlorpyrifos to skeletal deformities in fish.

Metals: Bioavailable heavy metals samples were collected using diffusion gel thin-films (DGT) and samples were deployed the same as the PSD. Bioavailable zinc, cadmium, lead and copper were determined for all 6 sampling campaigns. Zinc, cadmium, lead, copper and arsenic (III) were determined in filtered grab water samples pulled during the 2003 sampling deployments. The bioavailable metal concentrations were, for the most part, below detection limits. The only exception was detection of approx. 100 µg/L Zn in a single sample from NP. However, as this detection was never replicated and all other samples from the site were below detection limits, this was considered an artifact. The results provided no evidence that bioavailable heavy metals were a likely cause for the difference in deformity loads at the two sites.

Maternal Transfer of POPs

Concentrations of POPs detected in Willamette River NPM ovary/oocyte tissue were relatively low. Chlorinated pesticide concentrations were generally less than 3.3 ng/g wet wt and only three of the 21 different chlorinated pesticide residues analyzed in Willamette River NPM ovary/oocyte tissue were detected (Table 3). Of the three, 4,4'-DDE was detected with the greatest frequency and at the greatest concentrations (Table 3). Endrin and 4,4'-DDD were detected in two of the ten samples analyzed, both from fish collected from the NP region. Exposure to ppb concentrations of toxic chlorinated pesticides similar to those detected in the ovary/oocyte tissue from some of the fish analyzed, have caused adverse effects in early life stage fish (Harris et al. 1994; Villalobos et al. 2003; PAN Pesticide Database). Thus, some potential for toxic effects of maternally transferred chlorinated pesticides was possible, although more extensive study would be needed to determine how probable such effects are.

Concentrations of maternally transferred PCBs detected in NPM ovary/oocyte samples were not alarming. A total of seven different PCB congeners were detected in one or more of the NPM ovary/oocyte samples (Table 3). PCB 153 was detected the most frequently (6/10 samples) and at the

greatest concentration, (up to 1.2 ng/g; Table 3). PCBs 110, 118, and 138 were each detected in a single ovary/oocyte sample from a fish collected from NP. All the PCB congeners detected were mono- or di-ortho substituted. These congeners tend to be much less toxic than the non-ortho planar PCBs (Harris et al. 1994; Walker and Peterson 1991; Zabel et al. 1995). Fish, in particular, were less sensitive to the mono-ortho PCBs than mammals or birds (van den Berg et al. 1998; Zabel et al. 1995). It was suggested that co-exposure to the relatively nontoxic mono and di-ortho PCB congeners reduced the overall uptake of the more toxic non-ortho planar PCBs (Harris et al. 1994). Mean concentrations of PCBs 8 and 18 were greater in fish collected from CV than those in fish from NP (Table 3). Conversely, the mean concentration of PCB 153 in ovary/oocyte tissue was greater in NP fish (Table 3). However, neither of these differences between sites were significant. Based on the samples analyzed, maternal transfer of PCBs was an unlikely risk for overt early life stage toxicity to Willamette River NPM.

PCDDs and PCDFs were detectable in all the ovary/oocyte samples analyzed. The specific congeners varied considerably among samples, therefore a toxic equivalents approach (van den Berg et al. 1998) facilitated analysis of the results and comparison among sites. Total 2,3,7,8-TCDD equivalents in oocyte/ovary tissue of Willamette River NPM ranged from 0.18 to 2.06 pg/g wet wt. The greatest TEQ concentration (2.06 pg/g wet wt) was detected in a fish from CV. However, the mean TEQ concentrations were nearly identical for fish collected from the two study sites (0.84 ± 0.20 versus 0.85 ± 0.31 pg/g wet wt for NP and CV, respectively; Table 3). Total concentrations of TEQs detected in NPM ovary/oocyte tissues were less than those expected to cause toxicity during early life stage development. Based on measured TCDD concentrations in fish eggs, the lowest observed effect concentration for 7 different fish species ranged from 270-2000 pg/g wet wt (Elonen et al. 1998). NOECs were greater than 175 pg/g wet wt (Elonen et al. 1998). The TEQ concentrations detected in this study were at least 135 times lower than the LOEC for the most sensitive of the seven species tested (Elonen et al. 1998). Furthermore, the TEQ concentrations detected were at least 2.5 times less than the probable no observable adverse effect level (NOAEL) of TEQs for lake trout, which is widely regarded

as the fish species most sensitive to dioxin (and dioxin-like) toxicity (Book et al. 2003; Elonen et al. 1998). Thus, concentrations of maternally transferred PCDDs and PCDFs unlikely caused early life stage toxicity in Willamette River NPM.

Based on the literature, it was unclear whether any of the concentrations of POPs detected in Willamette River NPM ovary/oocyte tissue likely caused skeletal deformities. However, no significant differences in maternally transferred POP concentrations were observed for fish from the NP versus CV study site (Table 3). Even if early life stage exposure to POPs was causing some disruption of early development, leading to skeletal deformities, it unlikely accounted for 2-3 fold greater rates of skeletal deformities among NP fish. As a whole, these results provided no compelling evidence that supported the hypothesis that greater maternal transfer of POPs was a likely cause for the greater prevalence of skeletal deformities in fish from the NP region of the Willamette River.

Sediment POPs

A small number (n=3 per site, per year) of surficial sediment samples collected from NP and CV sites were analyzed for persistent chlorinated pesticide residues, PCBs and organophosphate pesticides to determine whether tropic transfer of these compounds from sediment, or direct exposure of embryo-larval fish (particularly for broadcast spawners; Cunningham et al. 2004) accounted for differences in deformity loads at the two sites. Chlorinated pesticides were not detected in samples collected from CV or NP in either 2002 or 2003. In 2002, PCB 8 was detected in 2/3 CV samples and 1/3 NP samples. Concentrations of PCB 8 ranged from 1.3-6.6 ng/g. Additionally, PCB 128 was detected in a single CV sample and PCBs 18, 101, and 153 were detected in a single NP sample. Concentrations of these congeners ranged from 0.5 (PCB 101) to 3.8 (PCB 18). In 2003, only 2 congeners, PCB 101 and PCB 110, were detected. PCB 101 was found in one CV sample and two NP samples, at concentrations ranging from 0.37-1.2 ng/g. PCB 110 (1.1 ng/g) was detected in a single sample from CV. Organophosphate pesticides were not detected in sediments from NP or CV. Samples were not collected at identical locations each year, so it was not possible to determine whether differences in the

congeners detected were primarily the result of spatial versus temporal differences. Overall, however, the results did not provide compelling support for the hypothesis that chlorinated pesticide residues, PCBs or organophosphate pesticides present in surficial sediments were a likely cause for the greater skeletal deformity load in fish from the NP region, relative to those from CV.

Skeletal Deformities Bioassay

Laboratory exposures of FHM to Willamette River water extracts from d.2-d.6 post-hatch with subsequent grow-out to d. 28-30 post-hatch provided no evidence that unknown compounds or chemical interactions caused greater deformity loads observed in fish from certain regions of the Willamette River. Survival to d 6 post-hatch ranged from 83-100% in all trials and there were no significant differences among treatments ($p=0.202-0.754$), indicating that the extracts were not acutely toxic to larval FHM. Survival during the grow-out period was variable among replicates and among trials, ranging from 5-19 fish per replicate (17-63%). In all cases, a minimum of 20 fish, per treatment group were examined for deformities. It was not possible to determine whether fish that died during grow-out were deformed. None the less, the lack of a significant treatment-related effect on survival to the examination day ($p=0.425-0.980$) suggested mortality during grow-out was randomly distributed among replicates and did not obscure a treatment effect.

When simple dorsal-ventral curvature was included as a deformity, 5-25% of the fish examined were classified as deformed, although no treatment-dependent effect was observed ($p=0.834-0.929$). When the analysis was restricted to only those deformities characterized as “qualitatively similar to those observed in Willamette River fish” (as per categories defined by Cunningham et al. 2004), the incidence of deformities ranged from 0.8%-2% for the entire population surveyed in each trial. Given the total sample sizes of 210-397 fish per trial, this represented 2-8 individual fish. In all cases, the deformities were spread across treatments, such that no association with any particular treatment was evident.

The distribution of developmental scores was unaffected by treatment in the NP/CV and AI/CV trials ($p=0.255$, 0.470), with most fish having developmental scores greater than 3. In the WF/CV trial, fish from the 4XC group were significantly more developed than those from all other treatment groups ($p=0.024$). However, no concentration-dependence was evident. As a whole, there was no evidence that the Willamette River water extracts induced skeletal deformities or otherwise adversely affected larval fathead minnows exposed for 96 h from d 2 to d 6 post-hatch.

A negative response in the skeletal deformities bioassay can not rule out the possibility that chemicals in the extracts had the potential to induce deformities in cyprinid fish. As designed, the assay provided a reasonable screen for the potential of the river water extract to disrupt early life-stage developmental processes important for later formation of the ossified vertebral column. The assay was not designed as an effective screen for chemicals that acted through acute neuromuscular damage or disruption of vertebral calcification. A time-series for skeletal development in FHM held under assay conditions showed that as early as d 5 post-hatch nearly all fish had ossified skulls and initial vertebral formation as indicated by ossification of the anterior-most centra (unpublished results). Deformities were not induced at non-toxic concentrations of Cd, Se, and chlorpyrifos as positive controls (unpublished results). However, the assay detected dexamethasone-induced deformities, suggesting effectiveness for screening for some mechanisms of action (Warner et al. unpublished). Robust application of the method will require additional characterization of the detectable mechanisms of action, and further optimization to reduce mortality-related variability during grow-out.

Parasites

Skeletal lesions in fish from the Willamette River were strongly linked with metacercariae of a diagenic trematode, likely *Apophallus donicus* (Kent et al. 2004, Cunningham et al. 2004, Niemi and Macy 1974). An analysis of cleared and stained specimens collected from four locations along the Willamette River (including the NP, WF, and CV study sites) concluded that the probability of a

precaudal skeletal deformity was strongly dependent on the number of trematode cysts in the body ($p < 0.0001$) and the area collected ($p = 0.006$) (Cunningham et al. 2004). Species and fish size were not significant predictors (Cunningham et al. 2004). Trematodes were directly associated with 86.5% of 592 primary precaudal deformities detected in chiselmouth examined as part of the parasite investigation (Cunningham et al. 2004, Kent et al. 2004). In NPM, trematodes were directly associated with 46.3% of the lesions observed (Cunningham et al. 2004). Additionally, a *Myxobolus* sp., likely *Myxobolus cyprini*, was associated with a significant portion (36%) of NPM with histologically verifiable skeletal lesions (Kent et al. 2004). These results suggested that parasites likely caused skeletal lesions observed in Willamette River fish. However, based solely on examination of field collected specimens, it was not possible to determine whether parasites actually caused the lesions or whether fish with lesions were simply more vulnerable to infection.

Results of the laboratory infection studies convincingly demonstrated that vertebral deformities, consistent with those observed in Willamette River fish, were caused by trematode cercariae. Five separate exposure trials were conducted with FHM (a cyprinid species) ranging from 8-17 days old (post-hatch). Mortality was variable and often high in both cercariae-exposed (14-71%) and control fish (5-91%). None the less, conclusions were drawn. A high incidence of infection (80-100%) was observed in cercariae-exposed fish from all trials (Table 4). Infected fish exhibited a high incidence of vertebral deformities (70-93%; Table 4). Most of the lesions were directly associated with metacercariae (Figure 2), and nearly all trematodes were directly located along the vertebral column. The types of deformities observed were identical to those observed in field collected specimens (Kent et al. 2004, Cunningham et al. 2004) including extra spines, lordosis, fused vertebrae, and increased vertebral density (Figure 2). Metacercariae occurred directly appressed to or deep within vertebrae and were often associated with bone hypertrophy. In contrast to cercariae-exposed fish, only 7% of the control fish examined exhibited skeletal deformities (Table 4). Control deformities were characterized as curvature of the spine or fused vertebrae. The incidence of skeletal deformities observed in control

fish for the infection studies were consistent with background rates of skeletal deformities determined for lab-reared fathead minnows examined by fluorescence microscopy.

Replication of the vertebral deformities, observed in fish collected from the field, by exposure to *Apophallus donicus* under controlled laboratory conditions further demonstrated that this parasite was likely a major cause of the lesions observed in cyprinid fish from the Willamette River. This heterophyid digenean trematode exhibits broad host specificity, infecting many species in the family Cyprinidae as well as fish from several other families (Niemi and Macy 1974; Kent et al. 2004; Cunningham et al. 2004). As observed in both our laboratory and field studies, the parasite exhibited remarkable affinity for bone (Kent et al. 2004, Cunningham et al. 2004). Most of the metacercariae were associated directly with skeletal structures and were not found in the viscera. 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*. Infections by other metacercariae types were linked to vertebral anomalies. Muscle infections by *Bucephalus polymorphus* caused vertebral deformities in cyprinid fishes (Baturu 1980), and *Riberiara sp.* was suspected to be a major cause of supernumerary limbs and other vertebral changes in frogs in North America (Kaiser 1999). Thus, both empirical evidence and literature reports supported the conclusion that trematode parasites caused the skeletal deformities observed in Willamette River fish.

Future Investigation

Although parasitic infection was established as the likely cause of skeletal deformities in Willamette River fish, questions remain as to whether the spatial differences observed were due to natural factors or anthropogenic influences. Increased occurrence of trematode infections were linked to anthropogenic pollution and physical alteration of aquatic habitats caused by human activities (Lardans and Dissous 1998). Potential synergism between exposure to herbicides and pesticides and susceptibility of frogs to infection by metacercariae of *Ribeiroia sp.* and *Telochris sp.* was reported

(Kiesecker 2002). None of the chemical contaminants detected in this study were known to cause immune suppression or increase susceptibility to infection at the concentrations observed. However, the biological assays used in this study were not designed to test the interaction between exposure to parasites and exposure to complex mixtures of chemicals present in Willamette River water and/or sediment (i.e. Willamette River water or sediment extracts). This would be a useful step toward determining the potential role of chemicals in promoting susceptibility to the parasites. Alternatively, it is equally possible, that the spatial difference in deformity loads was reflective of natural phenomenon. Given the life cycle of *Apophallus* sp. (Niemi and Macy 1974), habitat characteristics favoring either the intermediate host (snails such as *Fluminciola* sp.) or the definitive host (fish-eating birds) could result in greater *Apophallus* sp. abundance, resulting in more infections. Natural factors influencing the viability, numbers, and microbial and other infectious agents, such as ORP, may also play a role. Additional study of the parasite ecology and potential interactions with anthropogenic influences could help determine appropriate management actions for affected regions of the Willamette River basin.

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Supporting Information Available: (1) List of all target analytes and parameters analyzed as part of *in situ* monitoring with a YSI 6920 Sonde probe and *in situ* sampling of bioavailable organic compounds

and metals using PSDs and EDTs, (2) diagram of float-cable-cage-cable-anchor setup, (3) 2002 and 2003 pH and ORP trends, (4) list of target analytes analyzed in ovary/oocyte tissues and surficial sediments and the concentrations detected in each sample, (5) developmental scoring criteria used for fathead minnow skeletal deformities assay, and (6) examples of deformities observed in fathead minnow skeletal deformities assay.

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FIGURE CAPTIONS

Figure 1. Diagram of the Willamette Basin depicting the general location and course of the Willamette River and primary study locations.

Figure 2. Photos showing various types of skeletal deformities and associated trematode metacercariae, observed in fathead minnows exposed to *Apophallus donicus* cercariae in the laboratory. *A. donicus* cercariae were isolated from *Fluminicola virens* snails collected from the NP region of the Willamette River.

Table 1. Frequency of occurrence of precaudal deformities and average deformity loads for the five species most commonly collected from three Willamette River study areas in 2002-2003.

Species	NP			Wheatland Ferry			CV		
	%	DL	N	%	DL	N	%	DL	N
<i>Ptychocheilus oregonensis</i>	23.6	0.37 ± 0.016	2314	22.8	0.35 ± 0.021	1205	6.9	0.08 ± 0.01	928
Northern pikeminnow		A			A			B	
<i>Richardsonius balteatus</i>	14.2	0.24 ± 0.029	515	13.0	0.20 ± 0.018	1091	6.3	0.09 ± 0.020	349
Redside shiner		A			A			B	
<i>Catostomus macrocheilus</i>	14.8	0.21 ± 0.015	1394	21.3	0.34 ± 0.111	47	17.3	0.22 ± 0.050	127
Largescale sucker		A			A			A	
<i>Mylocheilus caurinus</i>	14.1	0.20 ± 0.032	305	24.2	0.34 ± 0.033	442	8.4	0.10 ± 0.038	96
Peamouth		AB			B			A	
<i>Acrocheilus alutaceus</i>	39.6	0.70 ± 0.061	268	55.1	0.97 ± 0.103	109	12.8	0.17 ± 0.030	251
chiselmouth		A			B			C	

%; Frequency of occurrence of precaudal deformities

DL: deformity load; number of deformity categories present per individual; mean ± SE (SE is individual rather than pooled)

N: sample size

A,B,C: Different letters indicate significant difference between sites ($p \leq 0.05$) based on Bonferroni multiple range test.

Table 2. Mean concentrations (ng/L) of bioavailable organics estimated from concentrations accumulated in passive sampling devices (PSDs) exposed for 21 d at two sites (2 locations per site) along the Wilamette River.

Bioavailable	CV		NP	
	2002	2003	2002	2003
Σ PAH ^c	3.17 ± 1.47	3.22 ± 1.22	2.01 ± 0.34	1.72 ± 0.54
Phenanthrene	1.15 ± 0.60	0.58 ± 0.22	0.93 ± 0.27	0.375 ± 0.107
Anthracene ^a	1.32 ± 0.52	1.18 ± 0.90	0.43 ± 0.12	0.04 ± 0.02
Fluoranthene ^c	0.70 ± .40	0.43 ± 0.13	0.66 ± 0.07	0.29 ± 0.07
Σ PCB ^c	0.035 ± 0.054	0.043 ± 0.020	0.067 ± 0.074	0.074 ± 0.026
Σ DDT ^a	0.102 ± 0.127	0.160 ± 0.018	0.222 ± 0.086	0.211 ± 0.051
p,p'-DDT ^b	0.013 ± 0.020	0.046 ± 0.004	0.045 ± 0.023	0.050 ± 0.009
p,p'-DDE	0.028 ± 0.036	0.067 ± 0.011	0.071 ± 0.026	0.092 ± 0.030
p,p'-DDD ^c	0.069 ± 0.011	0.045 ± 0.007	0.106 ± 0.040	0.069 ± 0.018
Dieldrin ^a	0.037 ± 0.041	0.064 ± 0.009	0.103 ± 0.030	0.112 ± 0.028
Chlorpyrifos	NA	0.74 ± 0.51	NA	1.38 ± 0.33

Target analytes with concentrations < detection limit not shown. See supplementary materials for complete list of analytes.

^a Significant difference between sites, both years

^b Significant difference between sites, 2002 only

^c Significant difference between sites, 2003 only

Table 3. Concentrations of chlorinated pesticides, polychlorinated biphenyls (PCBs), and polychlorinated dibenzo-p-dioxins (PCDDs) and polychlorinated dibenzofurans (PCDFs) detected in oocyte/ovary tissue from northern pikeminnow (*Ptychocheilus orgeonensis*) collected from NP (NP) and CV (CV) study sites.

Compound	Mean \pm SE (ng/g) ^a		Median (ng/g) ^a		P
	NP	CV	NP	CV	
Endrin	3.94 \pm 1.62	<i>1.62\pm0.01</i>	1.65	1.62	0.191
4,4'-DDD	3.63 \pm 1.34	<i>1.62\pm0.01</i>	1.65	1.62	0.171
4,4'-DDE	46.9 \pm 13.4	48.0 \pm 15.9	35.0	31.0	0.942
PCB-8 [2,4']	3.26 \pm 1.74	10.8 \pm 4.68	1.65	7.70	0.144
PCB-18 [2,2',5]	<i>1.55\pm0.07</i>	16.7 \pm 15.1	1.65	1.65	0.999
PCB-101 [2,2',4,5,5']	3.52 \pm 0.90	<i>2.21\pm0.06</i>	4.16	1.65	0.402
PCB-110 [2,3,3',4',6]	<i>1.98\pm0.46</i>	<i>1.62\pm0.01</i>	1.65	1.62	0.674
PCB-118 [2,3',4,4',5]	<i>2.48\pm0.96</i>	<i>1.62\pm0.01</i>	1.65	1.62	0.674
PCB-138 [2,2',3,4,4',5']	<i>2.80\pm1.27</i>	<i>1.62\pm0.01</i>	1.65	1.62	0.674
PCB-153 [2,2',4,4',5,5']	4.20 \pm 0.92	<i>2.65\pm0.65</i>	4.20	1.65	0.163
TEQ [PCDD/DFs] (pg/g wet wt)	0.84 \pm 0.20	0.85 \pm 0.31	0.99	0.67	0.959

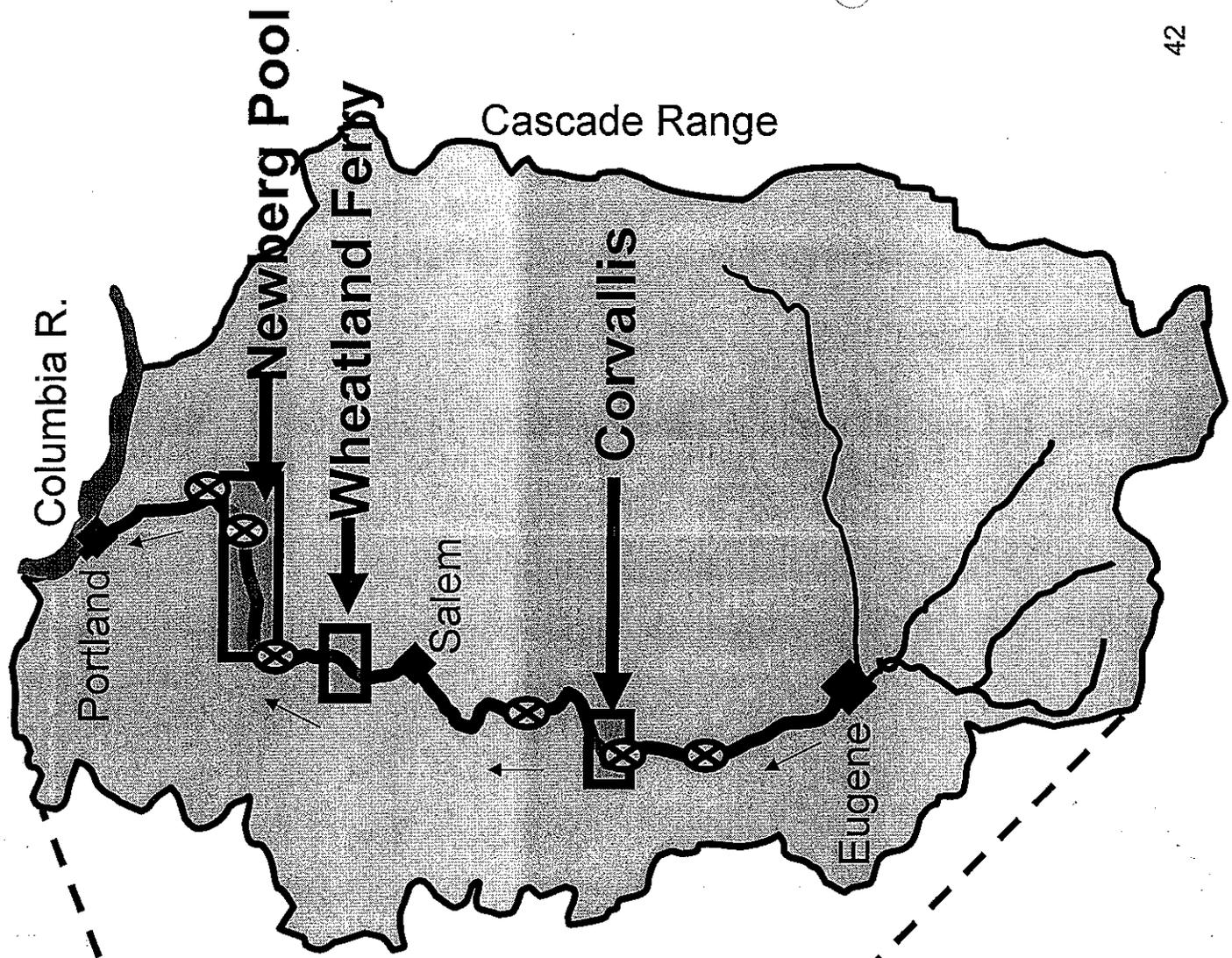
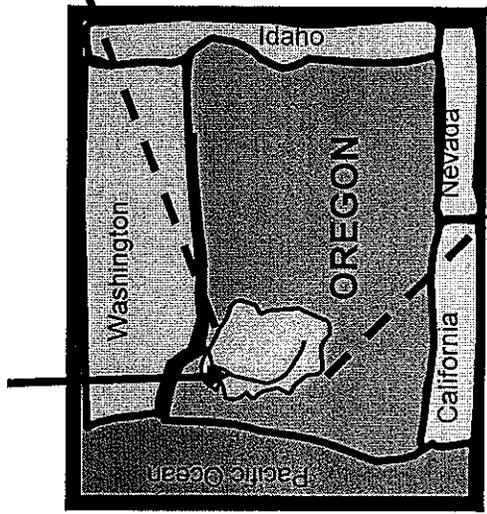
^a For the purposes of calculating means, medians, and statistics, non-detects were assumed to be equal to ½ the method detection limit (MDL). All concentrations reported in ng/g wet wt. except TEQ which are reported as pg/g wet wt.

Italics indicate that mean or median estimate was less than the MDL.

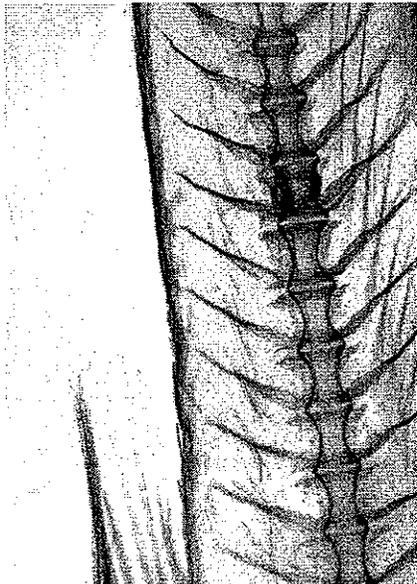
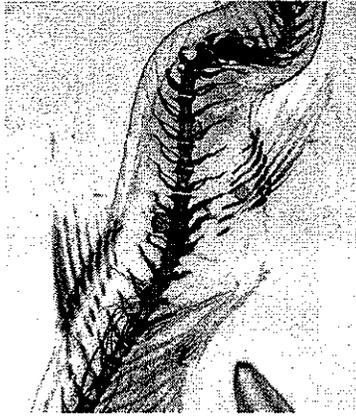
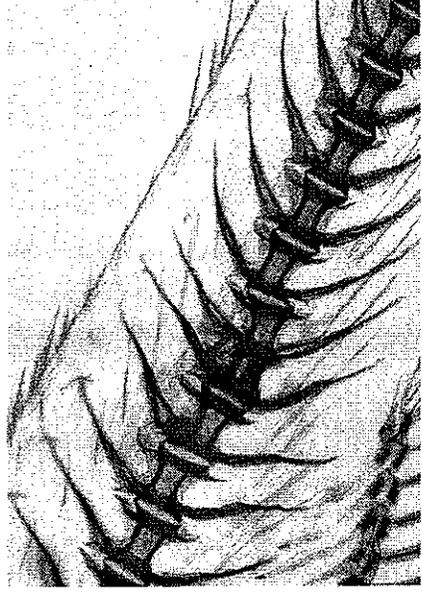
Table 4. Incidence of vertebral deformities and metacercariae in fathead minnows (*Pimephales promelas*) exposed to cercariae of *Apophallus* donicus.

Trial #	Concentration of Exposure	Age of Exposure (days)	Days Post Exposure when Examined	Number Examined	% Deformed	% Infected	Abundance	% lesions associated with parasites	% worms asso. With lesions
1	30	8	55	11	8/11 (73)	9/11 (82)	1.0	9/10 (90)	9/11 (82)
1C	0	8	55	7	0	0	0	NA	NA
2	10	8	70	14	12/14 (86)	13/14 (93)	1.9	17/17 (100)	20/27 (74)
2C	0	8	70	18	1/18 (6)	0	0	0	NA
3	30	5	70	10	7/10 (70)	8/10 (80)	1.2	8/9 (88)	8/12 (67)
3C	0	5	70	12	0	0	0	NA	NA
4	30	17	70	14	13/14 (93)	14/14 (100)	4.5	31/33 (94)	38/64 (59)
5	30	24	70	21	19/21 (91)	20/21 (95)	4.0	37/42 (88)	58/88 (66)
4/5C	0	17/24	70	18	1/18 (6)	0	0	0	NA
totals exposed				70	84	91	2.5	93	66
controls				55	4	0	0	0	0

The Willamette Basin



	major tributary enters river
	Primary study sites
	City locations for geographic reference
	Direction of flow





APPENDIX I

SUPPORTING INFORMATION



2

3 **Table SI-1:** List of all target analytes and parameters analyzed as part of *in situ* monitoring with a YSI
 4 6920 Sonde probe and *in situ* sampling of bioavailable organic compounds and metals using passive
 5 sampling devices (semi-permeable membrane devices-SPMDs) and diffusion gradient thin films
 6 (DGTs).

7

PAHs	Organochlorine Pesticides	Polychlorinated Biphenyls
naphthalene	p,p'-DDT	37; 3,4,4'-TrCB
acenaphthylene	p,p'-DDD	44; 2,2',3,5'-TeCB
acenaphthene	p,p'-DDE	49; 2,2',4,5'-TeCB
fluorene		52; 2,2',5,5'-TeCB
phenanthrene		60; 2,3,4,4'-TeCB
anthracene	Organonitrogen Pesticides:	74; 2,4,4',5'-TeCB
fluoranthene	s-Metaloclor	77; 3,3',4,4'-TeCB
pyrene	Napropamide (Devrinol)	87; 2,2',3,4,5'-PeCB
benzo(a)anthracene		99; 2,2',4,4',5'-PeCB
chrysene		101; 2,2',4,5,5'-PeCB
benzo(b)fluoranthene		105; 2,3,3',4,4'-PeCB
benzo(k)fluoranthene,		114; 2,3,4,4',5'-PeCB
benzo(a)pyrene,		118; 2,3',4,4',5'-PeCB
dibenzo(a,h)anthracene,	Organophosphate Pesticides	126; 3,3',4,4',5'-PeCB
benzo(g,h,I)perylene,	Dimethoate	128; 2,2',3,3',4,4'-HxCB
indenol(1,2,3-cd)pyrene	Diazinon	138; 2,2',3,4,4',5'-HxCB
	Chlorpyrifos	153; 2,2',4,4',5,5'-HxCB
Physico-chemical	Azinphos-methyl (Guthion)	156; 2,3,3',3,4,4',5-HxCB
pH		166; 2,3,4,4',5,6-HxCB

Dissolved oxygen		169; 2,3',4,4',5,5'-HxCB
Nitrate	Metals	170; 2,2',3,3',4,4',5-HpCB
Ammonia	Lead	180; 2,2',3,4,4',5,5'-HpCB
Oxidative reduction potential	Zinc	183; 2,2',3,,4,4',5',6-HpCB
Temperature	Cadmium	187; 2,2',3,4',5,5',6-HpCB
Specific conductance	copper	189; 2,3,3',4,4',5,5'-HpCB

1

2 TrCB = trichlorobiphenyls

3 TeCB = tetrachlorobiphenyls

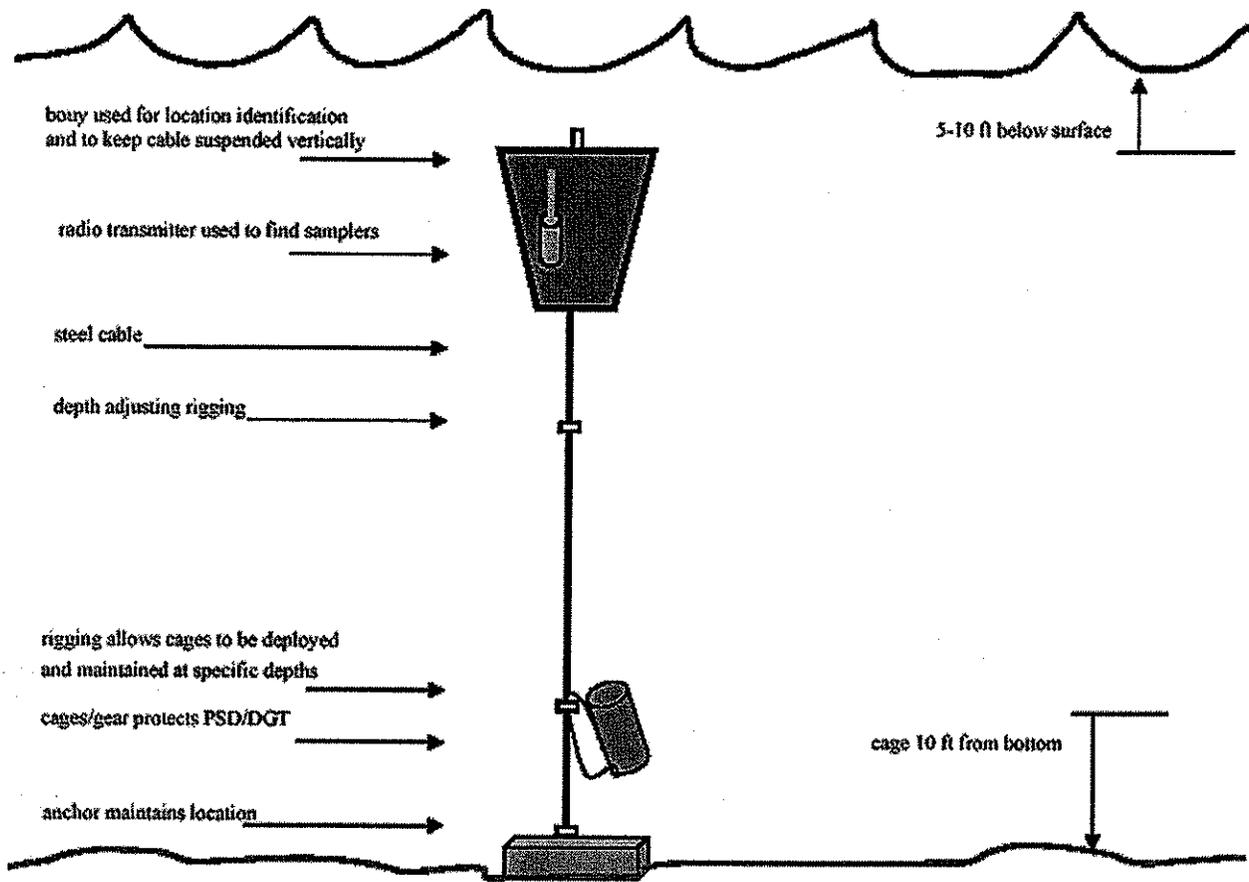
4 PeCB = pentachlorobiphenyls

5 HxCB = hexachlorobiphenyls

6 HpCB = heptachlorobiphenyls

7

1 **Figure SI-1:** Diagram of boat-cable-cage-cable-anchor setup used for *in situ* monitoring and
2 sampling.



3 Not drawn to scale

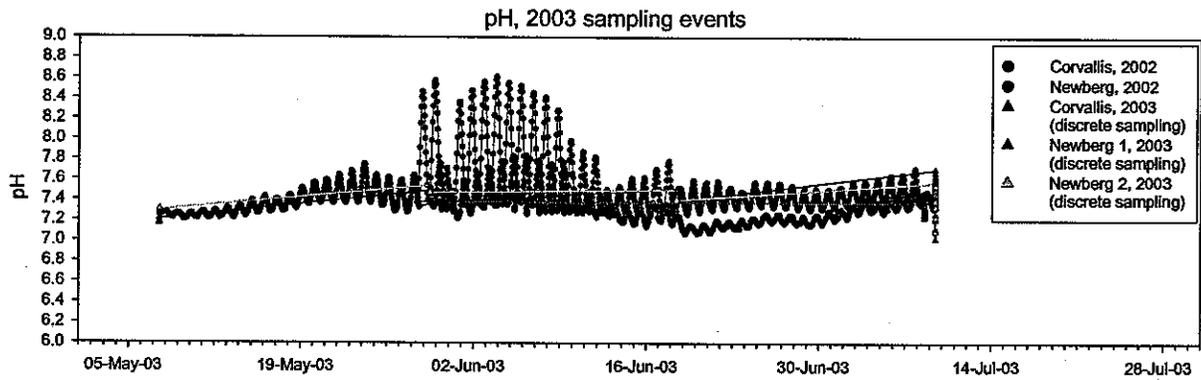
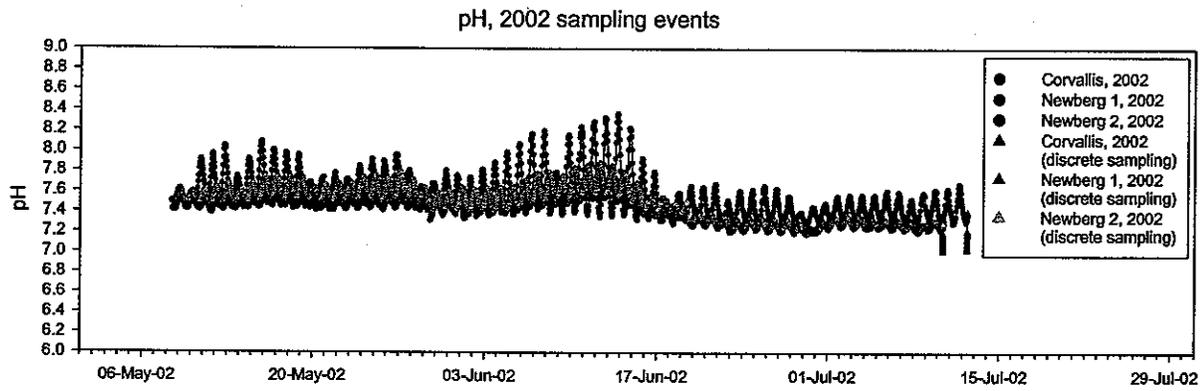
Anderson, K.A. schematic of PSD deployment setup & gear

4

5

1 **Figure SI-2: pH at each station, for 2002 and 2003.**

2



3

4

5

1 **Figure SI-3:** Oxidation-reduction potential (ORP) at each station for 2002 and 2003.

2

3

4

5

1 **Table SI-2:** List of all target analytes analyzed for the study of persistent organic pollutants in
 2 ovary/oocyte tissue from Willamette River northern pikeminnow (*Ptychocheilus oregonensis*) and
 3 surficial sediment.

4
5

Chlorinated Pesticides ^a	Polychlorinated Biphenyls ^a		PCDDs and PCDFs ^b
	Substitution Pattern	BZ #	
Alpha-BHC	2,4'-di	8	2,3,7,8-TCDD
Gamma-BHC (lindane)	2,2',5-tri	18	1,2,3,7,8-PeCDD
Beta-BHC	2,4,4'-tri	28	1,2,3,4,7,8-HxCDD
Delta-BHC	2,2',5,5'-tetra	52	1,2,3,6,7,8-HxCDD
Heptachlor	2,2',3,5'-tetra	44	1,2,3,7,8,9-HxCDD
Aldrin	2,3',4,4'-tetra	66	1,2,3,4,6,7,8-HpCDD
Heptachlor epoxide	2,2',4,5,5'-penta	101	OCDD
Alpha chlordane	3,4,4',5-tetra	81	
4,4'-DDE	2,3,3',4',6-penta	110	2,3,7,8-TCDF
Endosulfan I	3,3',4,4'-tetra	77	1,2,3,7,8-PeCDF
Dieldrin	2,3,4,4',5-penta	123	2,3,4,7,8-PeCDF
Endrin	2,3',4,4',5-penta	118	1,2,3,4,7,8-HxCDF
4,4'-DDD	2,2',4,4',5,5'-hexa	153	1,2,3,6,7,8-HxCDF
Endosulfan II	2,3,3',4,4'-penta	105	1,2,3,7,8,9-HxCDF
4,4'-DDT	2,2',3,4,4',5'-hexa	138	2,3,4,6,7,8-HxCDF
Endrin aldehyde	2,2',3,4,4',5,5',6-hepta	187	1,2,3,4,6,7,8-HpCDF
Methoxychlor	3,3',4,4',5-penta	126	1,2,3,4,7,8,9-HpCDF
Endosulfan sulfate	2,2',3,3',4,4'-hexa	128	OCDF
Endrin ketone	2,3',4,4',5,5'-hexa	167	
Chlordane	2,3,3',4,4',5-hexa	156	
Toxaphene	2,3,3',4,4',5'-hexa	157	

	2,2',3,3',4,4',5,5'-hepta	180	
	2,2',3,3',4,4',5-hepta	170	
	3,3',4,4',5,5'-hexa	169	
	2,3,3',4,4',5,5'-hepta	189	
	2,2',3,3',4,4',5,6-octa	195	
	2,2',3,3',4,4',5,5',6-nona	206	
	-deca	209	

1 ^a Method reporting limits (MRL) for chlorinated pesticides and PCBs ranged from 2.5 - 3.3 µg/Kg wet
2 wt for ovary/oocyte tissue and 0.33 µg/Kg wet wt. for sediment. Determined for both ovary/oocyte and
3 sediment samples.

4 ^b PCDD = polychlorinated dibenzo-*p*-dioxin; PCDF = polychlorinated dibenzofuran; T=tetra (4 Cl
5 substitutions); Pe = penta; Hx =hexa; Hp=hepta; O=octa. Detection limits for PCDDs & PCDFs ranged
6 from 0.10 – 0.13 ng/Kg wet wt. Determined for ovary/oocyte tissue only.

7

1 **Table SI-3.** Summary of concentrations of persistent organic pollutants detected in ovary/oocyte
 2 tissue from fish collected from the Newberg Pool or Corvallis region of the Willamette River.
 3 Concentration units are ng/g wet wt for chlorinated pesticides and PCBs. Units are pg/g wet wt for
 4 TEQs.

Compound	NP1	NP2	NP3	NP4	NP5	C1	C2	C3	C4	C5
Endrin	nd	nd	nd	5.72	9.53	nd	nd	nd	nd	nd
4,4'-DDD	nd	nd	nd	6.17	7.56	nd	nd	nd	nd	nd
4,4'-DDE	28.0	14.6	35.0	67.7	86.9	31.0	22.7	53.1	108	25.2
PCB-8 [2,4']	nd	nd	nd	nd	10.2	7.70	28.2	nd	4.49	12.0
PCB-18 [2,2',5]	nd	nd	nd	nd	nd	nd	nd	nd	76.9	nd
PCB-101 [2,2',4,5,5']	4.16	nd	nd	4.48	6.03	nd	nd	4.56	nd	nd
PCB-110 [2,3,3',4',6]	nd	nd	nd	nd	3.81	nd	nd	nd	nd	nd
PCB-118 [2,3',4,4',5]	nd	nd	nd	nd	6.32	nd	nd	nd	nd	nd
PCB-138 [2,2',3,4,4',5']	nd	nd	nd	nd	7.89	nd	nd	nd	nd	nd
PCB-153 [2,2',4,4',5,5']	nd	4.2	4.19	5.68	11.18	4.71	3.68	nd	nd	nd
TEQs [PCDDs + PCDFs]	0.59	0.18	1.09	0.99	1.34	0.59	0.67	2.06	0.73	0.24

6 NP = samples from fish collected at the Newberg Pool study site

7 C = samples from fish collected at the Corvallis study site

8 nd – indicates concentration was < the MRL or detection limit

9

10

1 **Table SI-4.** Summary of concentrations of persistent organic pollutants detected in surficial sediment
 2 samples from the Newberg Pool (NP) or Corvallis (C) region of the Willamette River. Concentration
 3 units are ng/g wet wt for chlorinated pesticides and PCBs.

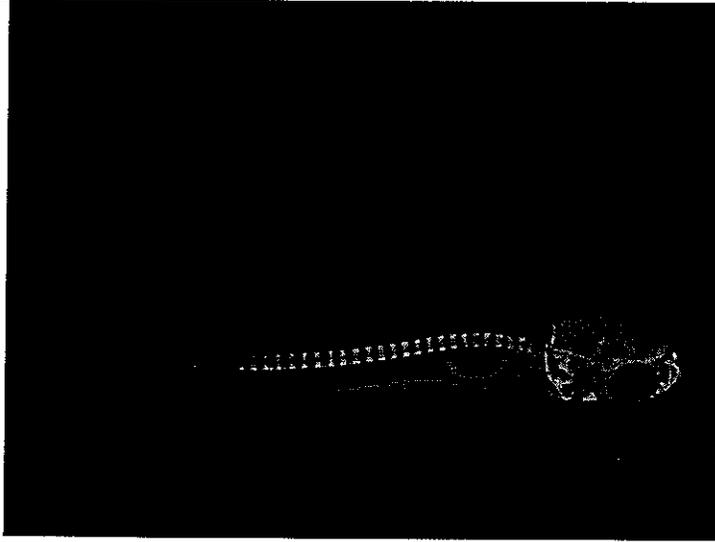
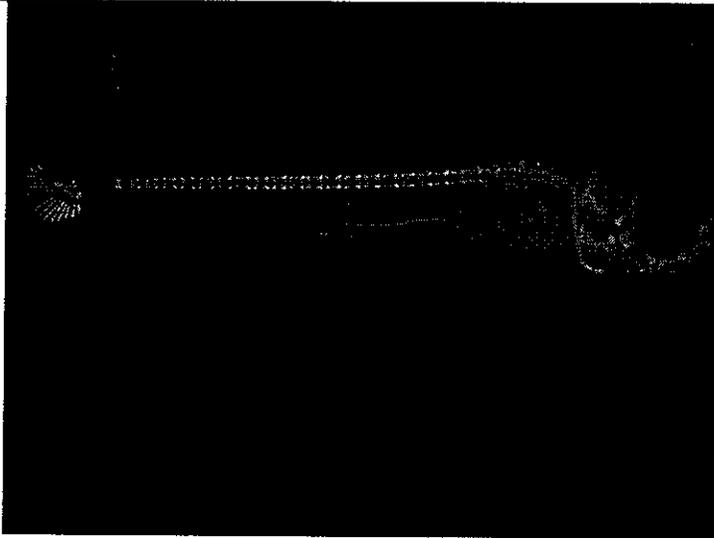
Compound	2002 ^a			2002 ^a		
	NP1	NP2	NP3	C1	C2	C3
PCB-8 [2,4']	nd	nd	4.6	6.6	1.3	nd
PCB-18 [2,2',5]	3.8	nd	nd	nd	nd	nd
PCB-101 [2,2',4,5,5']	nd	nd	nd	nd	nd	0.50
PCB-128 [2,2',3,3',4,4']	nd	nd	nd	nd	nd	0.7
PCB-153 [2,2',4,4',5,5']	nd	nd	nd	nd	nd	1.2
Compound	2003 ^b			2003 ^b		
	NP1	NP2	NP3	C1	C2	C3
PCB-101 [2,2',4,5,5']	nd	0.37	1.2	0.73	nd	nd
PCB-110 [2,3,3',4',6]	nd	nd	nd	nd	nd	1.1

4 ^a Sampling locations: NP- N 45°16.308, W 122°59.460; C- N 44°31.567, W 123°15.373

5 ^b Sampling locations: NP- N 45°15.567, W 122°54.231; C- N 44°32.887, W 123°15.432

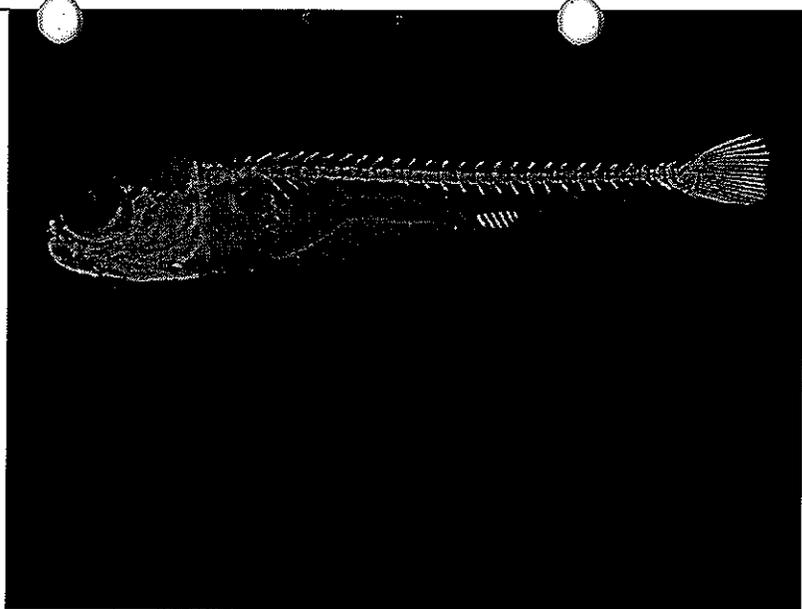
6
7
8

- 1 **Table SI-5.** Fathead minnow skeletal deformities bioassay, developmental scoring criteria with
2 example fluorescence micrographs. Age of all fish shown was 24 days post-hatch.

<p>Developmental score = 1</p> <p>Vertebral ossification anterior to pelvic fins only. Little or no fin ray development.</p>	
<p>Developmental score = 2</p> <p>Incomplete spinal development. Vertebral development extends past pelvic fin region but is not complete all the way to caudal fin. Moderate fin ray development.</p>	

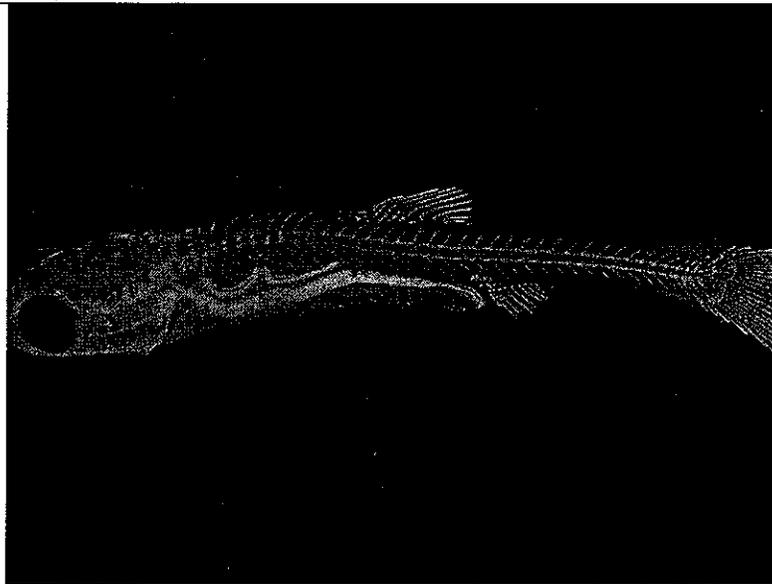
Developmental score = 3

Vertebrae fully developed from head to caudal fin. Not all vertebrae have visible, paired neural and haemal spines. Ossified ribs not visible. Incomplete fin ray development.



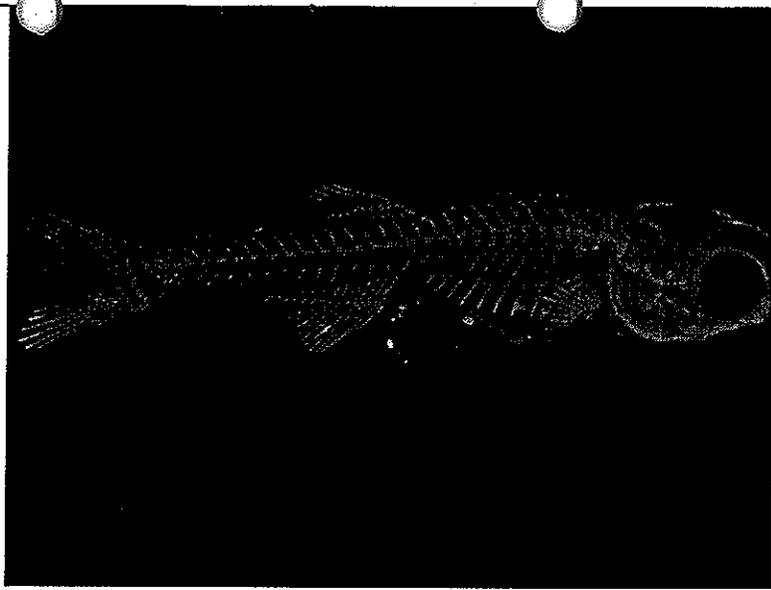
Developmental score = 4

Vertebrae fully developed from head to caudal fin. Neural and haemal spines visible and present on all vertebrae. Ossified ribs visible. Moderate fin development.



Developmental score = 5

Fully developed skeletal structure from head to caudal fin. Fully developed complement of fin rays evident.



1

2

1 **Table SI-6. Examples of deformities observed in the fathead minnow skeletal deformities bioassay.**

2

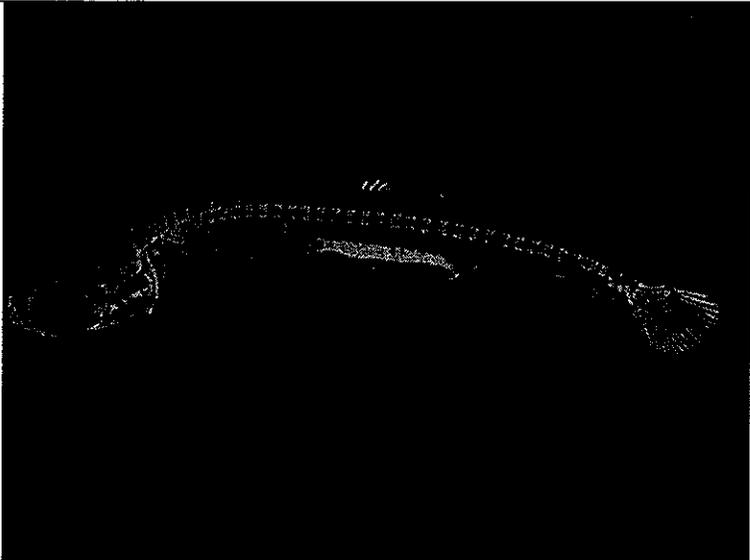
<p>Photo 6-1^a</p> <p>Fathead minnow with dorsal curvature</p> <p>d. 24 post-hatch</p> <p>Control</p>	
<p>Photo 6-2^a</p> <p>Fathead minnow with curvature over swim bladder</p> <p>30 d. post-hatch</p> <p>Exposed to 8X-Corvallis extract</p>	

Photo 6-3^b

Fathead minnow with
multiple spine
deformities and fused
vertebrae

d. 28 post-hatch

Exposed to 4X COR
extract

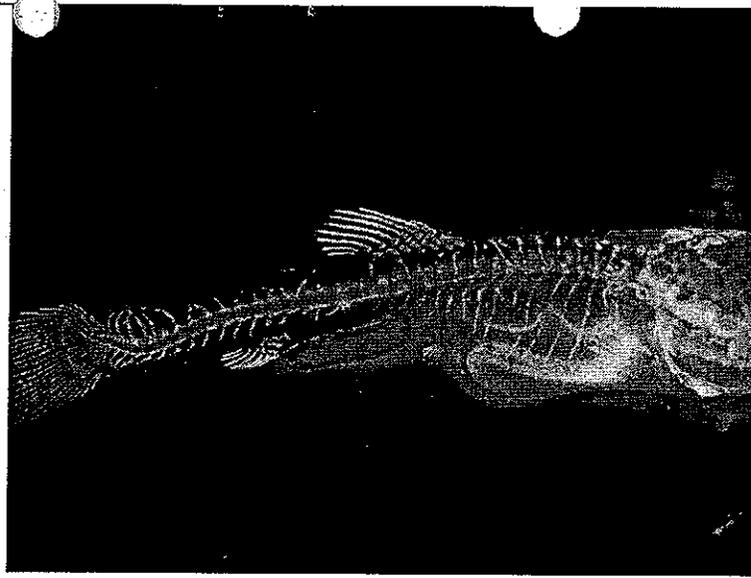


Photo 6-4^b

Close up

Fathead minnow with
compressed vertebrae.

d. 25 post-hatch

Exposed to 1X AI
extract

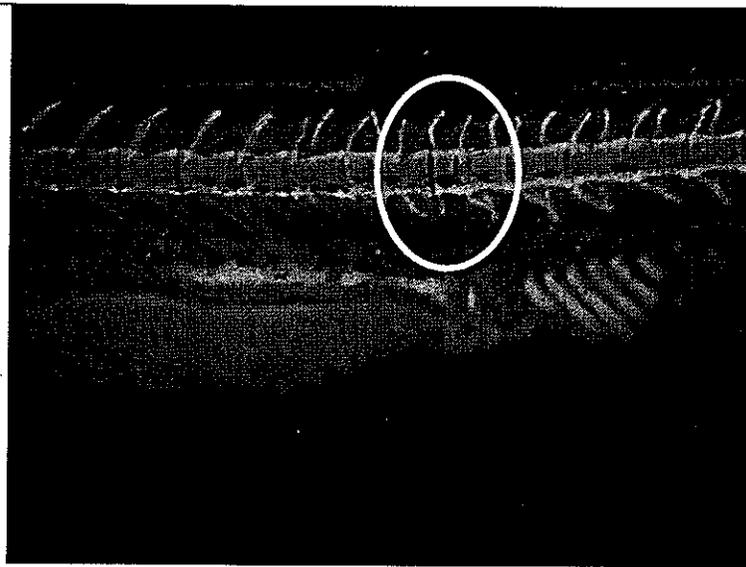
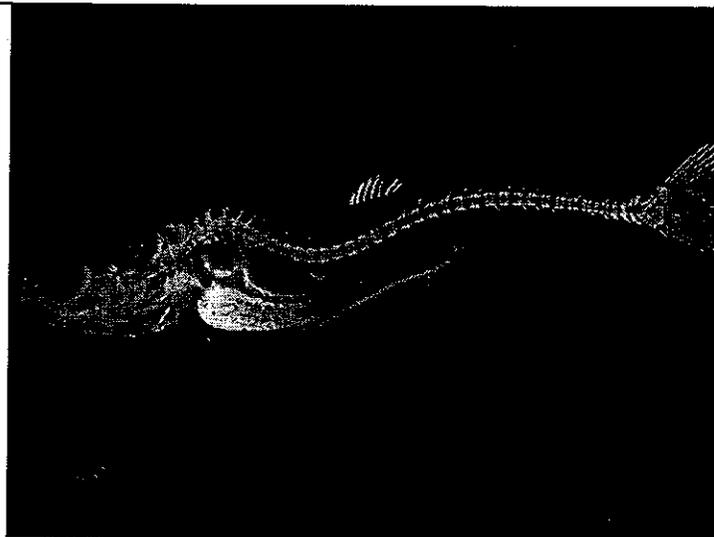


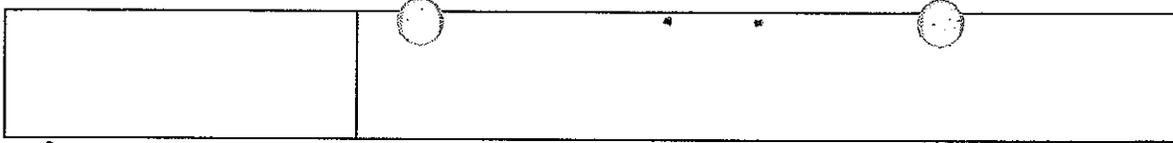
Photo 6-5^b

Fathead minnow with
severe lordosis

d. 25 post-hatch

Exposed to laboratory
dechlorinated tapwater
(control)





- 1 ^a Photos 6-1 and 6-2 characterized as minor deformities not consistent with deformities observed in
- 2 Willamette River fish.

- 3 ^b Photos 6-3 through 6-5 show deformities characterized as qualitatively similar to those observed in
- 4 Willamette River fish.



APPENDIX II

PATTERNS OF FISH DEFORMITIES AND THEIR ASSOCIATION
WITH TREMATODE CYSTS IN THE WILLMETTE RIVER



Patterns of fish deformities and their association with trematode cysts in the Willamette
River, Oregon

by

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Keywords: Fish deformities, trematodes, Willamette River, Oregon, northern
pikeminnow, chiselmouth, cyprinids.

Synopsis

Skeletal deformities in Willamette River fishes were described from over 16,000 larval and juvenile specimens collected in 2002 and 2003. Deformities were found in most taxa examined but were more frequent in native broadcast spawners, especially minnows and suckers, than in native or exotic nest builders. Caudal deformities were uniformly distributed throughout the river, but precaudal deformities were more localized near the towns of Newberg and Salem (Wheatland Ferry). In northern pikeminnow (*Ptychocheilus oregonensis*), susceptibility to deformities was dependent on relative birth date, with early season fish having about three times the deformity load as late season fish. In a subsample of northern pikeminnow and chiselmouth, number of deformities was directly related to number of trematode metacercariae and precaudal deformities were twice as likely as caudal deformities to be associated with metacercariae. A logistic regression demonstrated that the probability of a precaudal deformity was dependent on number of metacercariae and geographic area with the area effect disappearing as the number of cysts increased. A separate analysis showed that some types of deformities were unlikely to be associated with metacercariae. However, even in cases where metacercariae were unlikely to be associated with deformities, metacercariae were usually present elsewhere in the fish and an indirect effect could not be dismissed. The taxonomic, spatial and temporal patterns of skeletal deformities in Willamette River fishes may be due to differences in intermediate host (snail or fish) resistance/susceptibility to parasites, to differences in life history ecology, and/or to anthropogenic effects that are manifest in increased precaudal deformities in fishes near Newberg and Wheatland Ferry.

Introduction

Typically, fish populations are expected to have skeletal deformity rates of 2 to 5% (Gill and Fisk 1966, Wells and Cowan 1982). In the Willamette River, Oregon, fish vertebral deformities are unusually high, especially from river mile (RM) 26.5 to RM 55, an area known as Newberg Pool (Ellis et al 1997, Ellis 2000, Markle et al. 2002, Kent et al. 2004). Deformities have been found in 12 species and six families with chiselmouth (*Acrocheilus alutaceus*) showing the highest rates (Ellis 2000, Markle et al. 2002).

Historical samples of northern pikeminnow (*Ptychocheilus oregonensis*) also showed high and variable frequencies of deformities (41.1-83.3%) from 1952-2000 (Markle et al. 2002) with no obvious long-term trend. This was a time frame including serious water quality problems that were not reversed until about 1970 (Laenen and Dojlido 1997).

Additionally, one of three northern pikeminnow collected in 1855 and housed at the Smithsonian Institution (radiograph of USNM 198, personal observation) has a doubled neural spine deformity.

Recently, Kent et al. (2004) showed that metacercariae of a digenean trematode, *Apophallus* sp., and a histozoicmyxozoan, resembling *Myxobolus cyprini*, were closely associated with most skeletal deformities in Willamette River chiselmouth and northern pikeminnow in histological sections. Deformities were almost always associated with one parasite or the other and only 2 of 63 deformities were associated with both parasites, thus deformities caused by one parasite do not attract the other parasite. Deformity-parasite relationships also differed between species with over 95% of chiselmouth deformities due to trematode metacercariae. In histological sections of northern pikeminnow, 21 of 41 (51%) deformities were associated with metacercariae and 15 of

41 (37%) with *Myxobolus*. In trypsin-cleared and stained northern pikeminnow, 8 of 15 (53%) deformities were associated with metacercariae. Although sample sizes were small, they suggested that the number of deformities in cleared and stained specimens was related to number of metacercariae, not fish species, fish size, or location in river. Kent et al (MS) noted a trade-off between evaluation technique and parasite/deformity detection. Radiographs can be used to evaluate many specimens quickly, and detect deformities but not parasites. Histology can detect both, but is costly when applied to large samples sizes and misses about 25% of radiograph-detected deformities. Cleared and stained whole specimens are less costly than histological sections, and can be used to detect deformities and most metacercariae but not most *Myxobolus* sp. infections.

In the following, we use radiographs to describe taxonomic, spatial and temporal patterns of skeletal deformities in many species of Willamette River fishes in 2002 and 2003. We also use cleared and stained specimens of the two most abundant species, northern pikeminnow and chiselmouth, to evaluate the smallest size at which metacercariae infest fish, the relationships between metacercariae and deformities, and whether particular categories of deformities are more or less likely to be caused by metacercariae. Potential explanation(s) of these deformities must be able to account for the patterns we see and we therefore discuss the relationship of parasites to these patterns.

Methods

Larval and juvenile fish were sampled by beach seine, cast net and dip net from river kilometer (RK) 222 in Corvallis downstream to RK 76.5 in Newberg. We collected 77 samples from 15 May - 2 October 2002, and 57 samples from 9 May - 20 August 2003 (Fig. 1). We grouped samples into five river areas. Three were heavily sampled: Area 1-

Newberg (RK 76.5- 85) – 51 samples, Area 3- Wheatland Ferry (RK 116-119) –29 samples, and Area 5- Corvallis (RK 201-222) –29 samples (Fig. 1). Areas 2 (RK 88-110) –7 samples and 4 (RK 125-185) –18 samples, were between the main areas and were sampled once when we collected juveniles throughout the Willamette River over a four week period from 16 July – 12 August 2002.

Specimens were fixed in 10% buffered formalin for a minimum of two weeks. We radiographed 15,730 fish on a Faxitron MX-20 cabinet x-ray machine using AGFA Structurix D4 DW ETE industrial radiograph film and developed on a Kodak X-OMAT Model M6B developer. Images were inspected for deformities using a 10X or 15X (depending on fish size) microscope ocular or loop over a light table. We radiographed and analyzed 14,419 fish in 2002 and 1,311 in 2003. A random sample of 550 fish were read independently a second time. Reader error was not significant. For example, for 294 northern pikeminnow, there were no differences in total ($p=0.87$), precaudal ($p=0.86$) and caudal ($p=0.89$) deformity loads.

We examined the size frequency of the five most abundant species caught in 2002 and assigned each individual to one of two age classes: young-of-the-year to age class 0 and larger individuals to age class 1. Modal sizes were quite distinct (Fig. 2) with both age classes available sometime during the season, except for peamouth which were almost all age 0. For the most abundant species, northern pikeminnow, we further classified individuals into three cohorts based on an assumption of uniform linear growth (Fig. 3). Cohort 1 would have had the earliest birthdates and cohort 3 the latest birthdates.

Twelve skeletal categories were scored as present or absent for deformities: precaudal centra, precaudal neural spines, precaudal haemal spines, caudal centra, caudal

neural spines, caudal haemal spines, dorsal fin pterygiophores, anal fin pterygiophores, dorsal fin rays, anal fin rays, caudal fin rays, and spinal curvature. We use the term precaudal in an unconventional way to refer to all centra and associated structures anterior of preural centrum 2. Caudal centra were previously shown to be highly variable and to have little geographic signal (Markle et al 2002).

To identify metacercariae associated with deformities, we cleared and stained 524 specimens (326 northern pikeminnow – 50 from 2002 and 276 from 2003; 198 chiselmouth – 64 from 2002 and 134 from 2003) following the methods of Potthoff (1984). Most of these fish were not included in the radiograph samples. Fish were 10-43 mm with 90% between 12- 36 mm. Deformities were scored using the 12 skeletal categories used for radiographs plus four additional categories seen on cleared fish: pectoral fin rays, pelvic fin rays, pectoral fin pterygiophores and pelvic fin pterygiophores. Each deformity was also scored for deformity type and association with metacercariae. Deformity types were: fragmented/broken spine, deflected spine, shortened spine, spine with nodule or bump, missing spine, multiple spines, branching spine, origin of spine in odd location, centrum spines not joined, ossified debris, centrum fused/shortened, centrum oddly shaped, centrum with holes, calcified material around centrum, fin rays deformed, vertebral column bent, and calcified material around spines. When multiple deformities were found together, we scored both the primary deformity and peripheral or cascading deformities. We scored association of deformities and metacercariae conservatively by requiring metacercaria to be within one myotome of the deformity to be considered associated with it. Trematodes not associated with deformities were also recorded and scored based on location on the fish: head, gill, body or fins.

Our predictor variables (such as area, age, size and parasite abundance) and our response variables (types of deformities) were highly skewed and/or had unequal variance. Transformations, such as Box-Cox, only partly normalized the data. Because our sample sizes were very large, we took two analytical approaches. We used comparisons of means to illustrate patterns in the data and include 99% confidence intervals. For significance testing, we used logistic regression and classified individuals in terms of presence/absence of deformities. Logistic regression does not require normal distributions or equal variance within each group but does require relatively large sample sizes > 50 (Tabachnick and Fidell 1996). All p values reported were from analysis of deviance of the logistic regression. The software used for statistical and graphical analyses was Statgraphics Plus 5.0 (Statistical Graphics Corporation 1994-2000).

Results

Taxonomic patterns

Eighteen taxa were represented by >19 individuals (Table 1). We grouped four species of sculpins (*Cottus asper*, *C. gulosus*, *C. perplexus*, and *C. rhotheus*) due to small sample sizes and similar deformity patterns. An additional 7 taxa were less common and excluded from most analyses. Fourteen of the 18 taxa had frequencies of all deformities greater than 24.4% (Table 1). One exotic (banded killifish- *Fundulus diaphanous*), and three natives (sculpins- *Cottus* spp., threespine stickleback- *Gasterosteus aculeatus* and leopard dace- *Rhinichthys falcatus*) had frequencies less than 15.8% with threespine stickleback almost completely devoid of deformities (1 in 52 fish). The average number of deformities per taxon (load) also showed great variation with the total load ranging

from 0.02 – 0.89, the precaudal load from 0.02 – 0.45 and the caudal load from 0 – 0.43 (Table 1).

In total, exotic fishes had about half the deformity load as native fishes. For example, the mean total deformity load for all native fishes (0.59) was higher than the mean for all exotic fishes (0.29) and the probability of a deformity was significantly different ($p=0.02$). The primary difference we see in vulnerability to deformities is in spawning behavior. Most native species are broadcast spawners, except threespine stickleback and sculpins whose deformity loads are low (Table 1), while the exotics are substrate spawners and mostly nest builders, except banded killifish which are vegetation spawners and mosquitofish which are viviparous (Breder and Rosen 1966, Wydoski and Whitney 2003).

Spatial patterns

During the river-wide survey from 16 July – 12 August 2002, we collected 8267 juveniles (Area 1- 1459, Area 2-2777, Area 3-621, Area 4-1918, and Area 5- 1116).

Spatial patterns for total deformity load differed among species but the common pattern for all species was that Newberg (area 1) and Wheatland Ferry (area 3) had higher numbers of total and precaudal deformities than the other areas (Fig. 4). Caudal deformity load (Fig. 4C) did not differ among areas. The probability of a deformity was not related to area for caudal deformities ($p=0.36$) but was related for total and precaudal deformities ($p=0.0001$). Total fin deformity loads were always low; the mean load per individual was 0.003. Because of these patterns, we focus on precaudal deformities for subsequent analyses.

Age patterns

Age appeared to be related to deformity load in four of the five most abundant species (Fig. 5 - very few age 1 peamouth were caught (22) and they were excluded from this analysis). However, when age is added to our logistic model for precaudal deformities it is not significant for any of the species ($p= 0.08 - 0.33$) while area remains significant ($p<0.03$).

For the most abundant species, northern pikeminnow, we also examined cohort effects. Precaudal deformity load in northern pikeminnow declined from the earliest cohort to the latest cohort (Fig. 6A) with cohort 1 having over three times the number of deformity categories per individual as cohort 3. Areas 1 and 3 had about 3-4 times the number of deformities as areas 2, 4 and 5 (Fig. 6B). When cohort and area are included in a logistic model for northern pikeminnow, both are significantly related to the presence or absence of precaudal deformities ($p<0.001$).

Metacercariae associated with deformities

Trematode metacercariae were easily seen in cleared and stained northern pikeminnow and chiselmouth (Fig. 7). Trematodes were found in the smallest fish examined (four in a 10 mm northern pikeminnow) and there were as many as 10 in a 12 mm chiselmouth. For 524 northern pikeminnow and chiselmouth, the number of primary precaudal deformities was linearly related to the number of trematode cysts in the body (Fig. 8). The absolute number of trematodes and deformities was much greater in chiselmouth and the relationship much stronger. Numbers of cascading deformities showed a similar pattern.

Cleared and stained specimens came from four areas: 1 (315), 2 (21), 3 (109) and 5 (79). We examined relationships of number of primary precaudal deformities with

species, fish size, geographic area, and number of body trematode cysts in a backwards stepwise logistic regression. Neither species ($p=0.28$) nor size ($p=0.97$) was important. In the final model (Fig. 9), the probability of having a precaudal deformity was based on number of trematode cysts in the body ($p<0.0001$) and area ($p=0.006$).

Primary deformities in some areas of the body and some types of primary deformities were more likely associated with trematodes than others. The average deformity of vertebral centra and spines (including neural, haemal, finrays and pterygiophores) had about 0.5-0.6 trematodes while the average debris deformity was significantly higher (0.8) (Fig. 10A). Similarly, the average precaudal and fin deformity had about 0.75 trematodes while caudal deformities were significantly less likely to have trematodes (0.4) (Fig. 10B).

Primary precaudal deformities were examined in greater detail (Table 2). For northern pikeminnow, every primary deformity type with a sample size >10 , had an average of >0.25 trematodes with over half of the categories averaging >0.68 trematodes per deformity (Table 2). For chiselmouth, every primary deformity type with a sample size >10 , had an average of >0.45 trematodes with over half of the categories averaging >0.92 trematodes per deformity (Table 2). For northern pikeminnow, 46.3% of 281 primary precaudal deformities were associated with trematodes, and for chiselmouth, 86.5% of 592 primary precaudal deformities were associated with trematodes. Similar patterns are seen in the relationship between precaudal deformities and trematodes anywhere in the body (Table 3).

We examined the probability that primary precaudal and caudal deformities were associated with trematodes using a logistic regression including size, species, geographic

area, deformity type, and area of the body as predictors. The model was significant ($p < 0.0001$), the adjusted percent deviance explained was 48.1% and the likelihood ratio tests for all variables were significant: size ($p = 0.0001$), body area-precaudal or caudal ($p = 0.0001$), species – chiselmouth or northern pikeminnow ($p < 0.0001$), deformity type ($p < 0.0001$) and geographic area ($p = 0.02$). For both species, some deformities had very high probabilities of being associated with trematodes and others had very low probabilities. The deformity types least likely to be associated with trematodes were branching spines (code = 7) and centra spines not joined at tips (code = 9, neural and haemal arches unformed) (Table 2). However, 88.4% of specimens with branching spines had body trematodes elsewhere in the body and 68.3% of specimens with unformed arches had body trematodes elsewhere in the body. Both of these deformity types were also commonly seen (branching spines-21 and centra spines not joined- 64) as cascading deformities associated with a trematode in related deformities such as fused centra. Our sample sizes were too small to examine individual deformity types in more detail (Table 2) but exploratory stepwise logistic regressions showed combinations of species, parasite abundance, fish size and geographic area were significant predictors.

Discussion

Taxonomic patterns in deformities (Table 1) largely agree with those described previously (Markle et al 2002). The important patterns are the large number of taxa showing deformities and the disparity between the average deformity load of native fishes (0.59) and exotic fishes (0.29). Outliers among native fishes are threespine stickleback, sculpins and leopard dace which have much lower frequencies of deformities (Table 1). Although there are slight differences in timing, all species examined are

spring-summer spawners and typically reach peak spawning in May and June (Breder and Rosen 1966; Widoski and Whitney 2004). A more obvious difference in vulnerability to deformities seems to be spawning behavior. Most of the native species are broadcast spawners, except threespine stickleback and sculpins, while the exotics are substrate spawners and most are nest builders, except banded killifish and mosquitofish (Widoski and Whitney 2004). It is not clear why larvae of broadcast spawners would be more vulnerable to cercariae than larvae from nest builders or substrate spawners but perhaps the disparity is simply due to host specificity. Typical of heterophyid trematodes, *Apophallus donicus* from the Willamette River basin appears to have broad host specificity for its second intermediate host. Niemi and Macy (1974) reported infections in a variety of fish families (Cyprinidae, Salmonidae, and Catostomidae). The parasite is likely a native parasite (Niemi and Macy 1974) and native fishes may simply be more susceptible. The relationship of deformities to fish spawning mode may be spurious, but deserves further study within a larger ecological context.

Within a species, there can be temporal differences in vulnerability that may be interannual (Fig. 5) or seasonal. Seasonal differences are largely due to the seasonality of cercariae emergence from snails. Cercariae emerge from snails and infect fish following increases in water temperature in spring or summer (Steinuer and Font 2003; Ginetsinskaya 1988; Terhune et al. 1998). In our data, early-spawned northern pikeminnow had about three times the precaudal deformity load as late-spawned fish (Fig. 6A). This birthdate-specific pattern was only examined in northern pikeminnow in 2002, and might differ interannually depending on the cues for fish spawning and cercaria emergence. If generally applicable to other species, it suggests a temporal component to

deformities of the same magnitude as geographic area effects (Fig. 6B). Again, a better understanding of the pattern requires a larger ecological context including parasite, snail and fish life history cues.

Geographic patterns in deformities showed patchiness with highest precaudal deformity loads near Newberg and Wheatland Ferry (Figs. 4A & B, 6, 9). As in other studies (Ellis et al. 1997, Ellis 2000, Markle et al. 2002), the precaudal deformity load for northern pikeminnow was highest near Newberg, but for many species it was highest at Wheatland Ferry. The Wheatland Ferry area was not sampled by Ellis et al (1997) while Markle et al. (2002) had small sample sizes and did not examine spatial patterns for any species other than northern pikeminnow. Our data show that both Newberg and Wheatland Ferry have high rates of deformities relative to other areas and that the intermediate area (2) has significantly lower rates (Fig. 4). There is nothing obvious about the geography of the two areas that would seem related to this deformity pattern. Newberg and Wheatland Ferry are largely agricultural but on the margins of two cities, Newberg and Salem, while the intermediate area is agricultural (Hulse et al. 2002). Areas 4 and 5, like Newberg and Wheatland Ferry, are also in mixed agricultural/ urban areas near Albany and Corvallis, yet their deformity patterns are more like area 2. Thus, there is a patchy and unexplained geographic component to deformities.

The positive, linear relationship between number of precaudal deformities in a fish and number of trematode cysts in the body (Fig. 8) suggests that the temporal and spatial patterns are largely biological and that fish from Newberg and Wheatland Ferry have more deformities because they have a greater abundance of trematodes. The logistic model for northern pikeminnow and chiselmouth (Fig. 9), however, shows that

geographic area is still a significant, though less important, variable that consistently appeared in this study. For example, in these two species, 0-3 trematodes are more likely to produce precaudal deformities in Newberg (area 1) than Corvallis (area 5). With more than five trematodes per fish, a precaudal deformity is almost certain regardless of area (Figs. 8 and 9). There were also deformities that had a low probability of a trematode association (Table 2). We believe that many of these are caused by trematodes passing through parts of the body on their way to final encysting sites (Kent et al. 2004). We were very conservative in scoring deformity-trematode associations. In two of the deformity types least likely to be associated with a trematode (codes 7 and 9), cysts were present elsewhere in the body in >68% of specimens.

Before attributing most of these deformity patterns to trematodes, we must also consider the second deformity-causing parasite in the river, *Myxobolus* sp., which is not detectable using our methodology. Kent et al. (2004) showed that *Myxobolus*-caused deformities were independent of trematode-caused deformities. Thus, unexplained deformity patterns in this study, such as the higher number of northern pikeminnow deformities not associated with metacercariae (Table 3), could be due to *Myxobolus* sp. However, some of the patterns we see can not be attributed to *Myxobolus* sp. For example, *Myxobolus* sp. seems to be rare in chiselmouth (Kent et al. 2004), and an unlikely factor for most of its deformity patterns. Also, since deformities caused by the two parasites seem to be independent (Kent et al. 2004), the seasonal decline in precaudal deformities in northern pikeminnow (Fig. 6A) could be due to the biology of both parasites.

The fundamental unanswered questions are to explain the taxonomic, seasonal and geographic patchiness in deformities. To the extent that these questions are related to trematodes, the two questions are, "why is the life cycle of this trematode apparently so successful in two areas but not in otherwise similar areas upstream and downstream?" and "why is it more successful in native broadcast spawners?" Trematodes are known to cause skeletal deformities in vertebrates and in one study of frogs, skeletal deformities were related to synergistic effects of trematodes and extremely high concentrations of agricultural pollutants (Kleicker 2002). In this case, skeletal deformities in frogs were caused by trematode cysts, but the number of deformities increased if pesticides were present. An increase in blood eosinophils suggested that pollutants compromised the frog's immunocompetency and led to higher infestation and more deformities (Kleicker 2002). Since larvae as small as 10 mm were affected in this study, and since this may be the approximate size at which immunocompetency develops in cyprinids (Iwama and Nakanishi 1996), parasite resistance could be an important factor explaining taxonomic and geographic patterns in our data. The susceptibility/resistance dichotomy is best understood in molluscan schistosomiasis (Bayne et al 2001). In other studies, patchy distributions of parasites have been attributed to genetic resistance of intermediate snail hosts (Johnson 2000) and to resistance of intermediate fish hosts (Leberg and Vrijenhoek 1994).

Patchy distributions of parasites can also be ecological (Esch 1994, Reimchen and Nosil 2001). There are examples of metacercariae infections in fish causing mortality (Lemly and Esch 1984) and increased susceptibility to predation (Lafferty and Morris 1996). Changes in deformities from age 0 to age 1 (Fig. 5) could be due to differential

mortality of infected individuals or to interannual differences in infection rates. For the trematode to be successful, we would expect differential mortality of infected individuals if final hosts disproportionately consume infected individuals, as seen with bird predation of California killifish (*Fundulus parvipinnis*) infected with metacercariae (Lafferty and Morris 1996). In our study, the trematode's tendency to locate near vertebrae and to cause skeletal deformities (Kent et al. 2004) could contribute to increased vulnerability. Direct evidence would have to come from tracking cohorts or year classes over time. In northern pikeminnow, cohorts within a year had significantly different deformity rates, with those born earlier in the spawning season having about three times the precaudal deformity load as those born towards the end of the season (Fig. 6A). Understanding the spatial and temporal patterns of cercarial release is necessary to better understand whether ecological aspects of the parasite's life cycle are responsible for some or all of these patterns.

The taxonomic, spatial and temporal patterns of skeletal deformities in Willamette River fishes are complex. Yet, the patterns can be directly or indirectly attributed to parasites or interactions of parasites with an, as yet, unknown geographic correlate(s). Concurrent water quality work has failed to demonstrate any unusual pollutants that might cause these patterns of deformities (Curtis et al., personal communication, 2004). The two known deformity-causing parasites' life histories are not well known (Kent et al. 2004) and the possibility of increased susceptibility or local resistance of hosts to these parasites (tubificid worms, or fish for *Myxobolus* sp., and snails, fish, fish-eating birds or mammals for *Apophallus*) is unexplored. For the trematode, release of cercariae by snails and infection in fish would likely be optimal with high densities of snails and fish, as happens seasonally when larval fishes congregate in shallow margins

of streams and rivers. These infections happen early in life causing disruption in bone growth and skeletal deformities which clearly may benefit the parasite's life cycle. The possibility that the geographic patchiness is due to localized differences in the success of this life cycle is also unexplored. For example, greater abundance of parasites in fish from certain regions of the river may be due to density of infected snails, proximity to juvenile fish, and water flow patterns. We have also not excluded the possibility that an unknown anthropogenic agent predisposes the fish to the trematode infection. Jeney et al. (2002) reported that roach exposed to pulp and paper mill effluent were more susceptible to *Rhipidocotyle fennica* infections. Lafferty (1997) reviewed the association of aquatic parasites and pollution, and in many cases trematode infections are enhanced by eutrophication but not by other types of pollution.

Acknowledgments

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TABLE 1. Sample size (N), average number of deformity categories per individual (deformity load), average precaudal deformity load, average caudal deformity load, and frequency of individuals with any deformity collected in 2002 and 2003 from the Willamette River. Species with sample sizes <19 excluded. Type indicates spawning mode where B= broadcast spawner, N= substrate spawner and nest builder, V= substrate spawner without nest building, and L=viviparous. Spawning types from Widowski and Whitney (2004). Asterisks indicate exotic species.

Species	Type	Deformity Load				
		N	Total	Precaudal	Caudal	Frequency %
<i>Ptychocheilus oregonensis</i> northern pikeminnow	B	6402	0.64	0.25	0.38	43.9
<i>Richardsonius balteatus</i> reidside shiner	B	2795	0.43	0.15	0.27	29.6
<i>Catostomus macrocheilus</i> largescale sucker	B	2113	0.59	0.20	0.39	41.0
<i>Mylocheilus caurinus</i> peamouth	B	1566	0.59	0.20	0.38	41.6
<i>Acrocheilus alutaceus</i> chislemouth	B	1006	0.89	0.45	0.41	50.9
<i>Fundulus diaphanous</i> * banded killifish	V	541	0.22	0.03	0.18	14.2
Cyprinid larvae, 15-22 mm	B	168	0.43	0.13	0.30	32.1
<i>Micropterus salmoides</i> *	N	218	0.33	0.10	0.22	25.2

largemouth bass

<i>Rhinichthys osculus</i> speckled dace	B	183	0.39	0.08	0.31	27.9
<i>Micropterus dolomieu</i> * smallmouth bass	N	123	0.43	0.32	0.09	24.4
<i>Rh. cataractae</i> longnose dace	N?	84	0.74	0.31	0.43	42.9
<i>Cottus spp</i> sculpins	N	61	0.08	0.03	0.05	8.2
<i>C. platyrhynchus</i> mountain sucker	B	57	0.74	0.37	0.37	38.6
<i>Gasterosteus aculeatus</i> threespine stickleback	N	52	0.02	0.02	0	1.9
<i>Lepomis macrochirus</i> * bluegill	N	39	0.13	0.05	0.08	12.8
<i>Gambusia affinis</i> * western mosquito fish	L	26	0.46	0.08	0.38	30.8
<i>Percopsis transmontana</i> sand roller	B	23	0.35	0.09	0.26	26.1
<i>Rh. falcatus</i> leopard dace	B	19	0.26	0.05	0.21	15.8

Table 2. Relationships between mean number of trematodes at a deformity and deformity type for precaudal deformities in cleared and stained fish. Spines include neural, haemal, finrays and pterygiophores depending on the area of the body with the deformity.

Code number & description	northern pikeminnow		chiselmouth	
	N	Mean trematodes	N	Mean trematodes
1. Fragmented/broken spine	30	0.80	126	1.06
2. Deflected spine	16	0.69	20	0.65
3. Shortened spine	15	0.87	30	1.00
4. Spine with nodule/bump	14	0.93	62	1.00
5. Missing spine	41	0.68	36	1.03
6. Multiple spines	8	0	4	0
7. Branching spine	12	0.25	22	0.54
8. Odd spine location	6	0.17	6	0.67
9. Centra spines not joined at tips	58	0.07	22	0.45
10. Calcified debris	3	1.00	46	1.06
11. Centrum fused/shortened	35	0.34	136	0.92
12. Centrum oddly shaped	16	0.50	41	0.80
13. Centrum holes	9	0.67	10	0.70
14. Calcified material around centrum	8	0.25	7	1.00
15. Vertebral column bent	2	0	6	1.17
16. Calcified material around spines	8	0.62	18	1.00

Table 3. Relationships between precaudal deformities and any trematode parasite in the body for chiselmouth and northern pikeminnow. Parasites may or may not be directly associated with the deformities. Number in parentheses is percent of total for each species.

Species	N	Deformed		Not deformed	
		With parasite	Without parasite	With parasite	Without parasite
chiselmouth	198	163 (82.3)	2 (1.0)	18 (9.1)	15 (7.6)
northern pikeminnow	326	119 (36.5)	46 (14.1)	47 (14.4)	114 (35.0)

Figure captions

Figure 1. Map of middle and lower Willamette River showing locations of three main sample areas sampled in 2002 and 2003 and intermediate sample areas sampled in 2002. Inset shows location of Willamette basin in Oregon.

Figure 2. Modal sizes of northern pikeminnow by month . Smaller mode assigned age class 0 and larger mode assigned age class 1.

Figure 3. Northern pikeminnow cohort assignment by length and month in 2002. X-axis not scalable by date.

Figure 4. Relationships between deformity loads and area for all species (N=8267) during river-wide surveys, 16 July – 12 August 2002. A. total deformity load. B. precaudal deformity load, C. caudal deformity load. Star indicates mean and bars show 99% Bonferroni confidence interval.

Figure 5. Age class differences in precaudal deformity load. A. northern pikeminnow, $p=0.01$, B. chiselmouth, $p=0.005$, C. redbelt shiner, $p=0.07$, D. largescale sucker, $p=0.04$. Star indicates mean and bars show 99% Bonferroni confidence interval.

Figure 6. Precaudal deformity load in northern pikeminnow in 2002 and relationships to A) cohort in age 0, N=3720 and B) area, N=5341 including age 1+. Star indicates mean and bars show 99% Bonferroni confidence interval.

Figure 7. Association of metacercariae with A) fused centra and B) deformed neural spines and debris in northern pikeminnow. Arrows point to the round cyst of the trematode.

Figure 8. Relationship between total number of trematode cysts in the body and total number of primary precaudal deformities in 326 northern pikeminnow and 198 chiselmouth. Regression line and equation shown for illustration purposes.

Figure 9. Logistic regressions for precaudal deformities and number of trematode cysts in the body for river areas 1, 2, 3 and 5. Adjusted percent of deviance (30.9%), model $p < 0.0001$, model residual $p = 0.9498$.

Figure 10. Relationships between number of trematodes per deformity and A) general deformity type and B) body area of deformity. Each relationship shows its component effect with the effects of species and type or area removed in a multifactor analysis of variance.

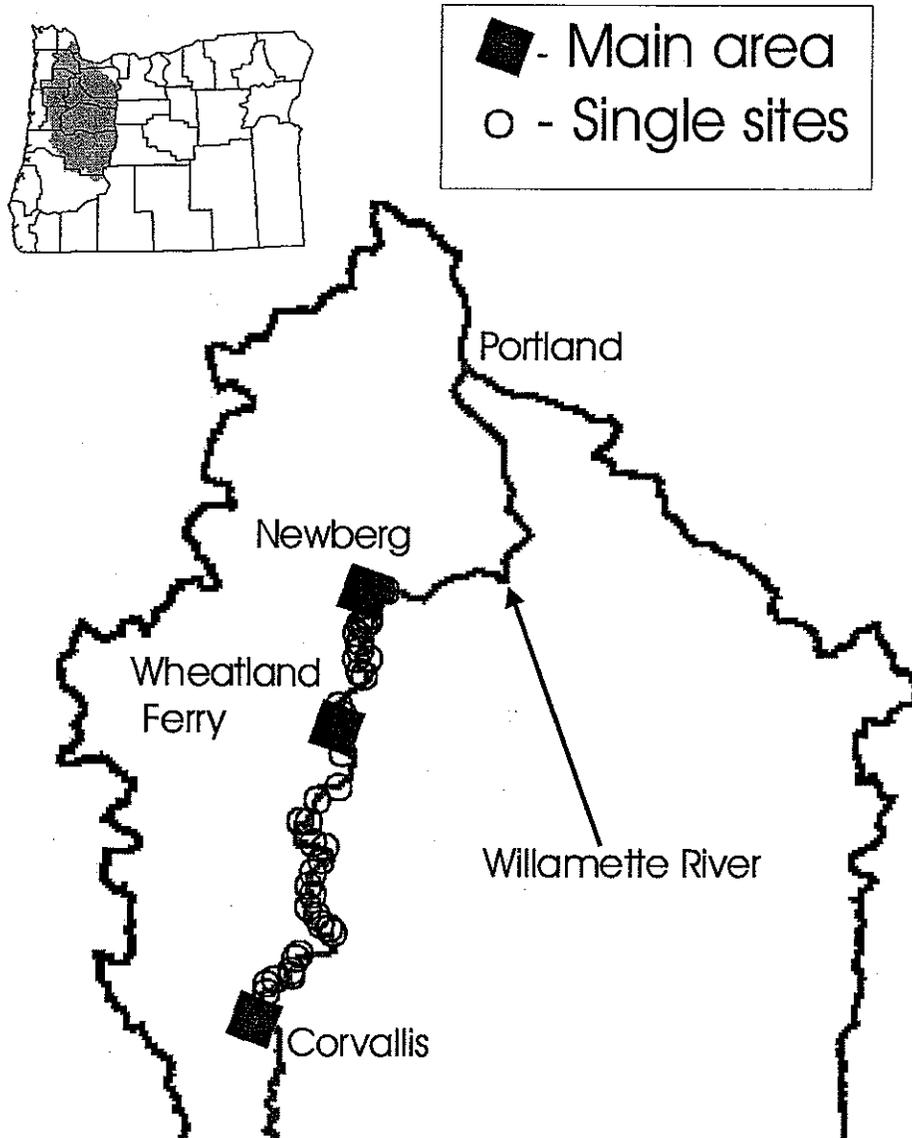


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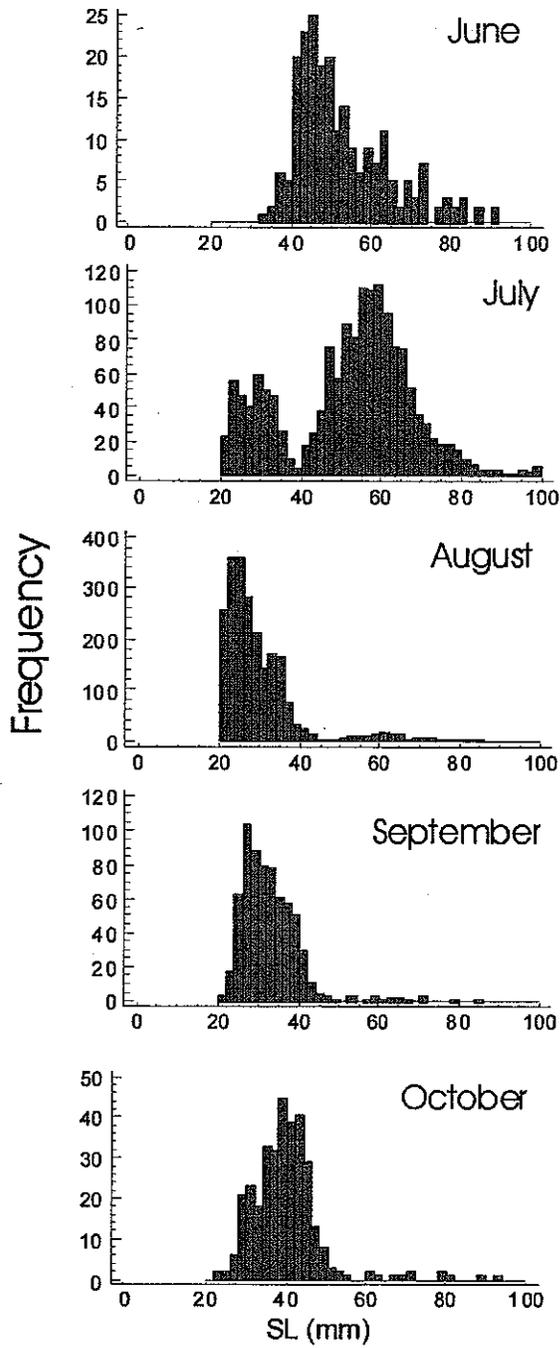


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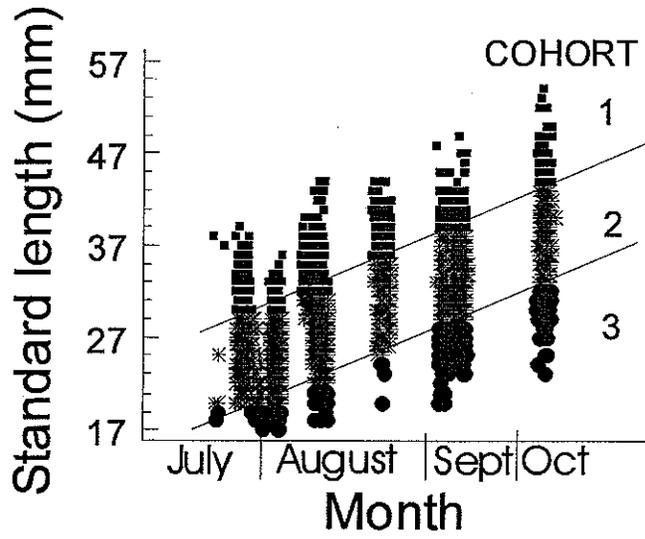


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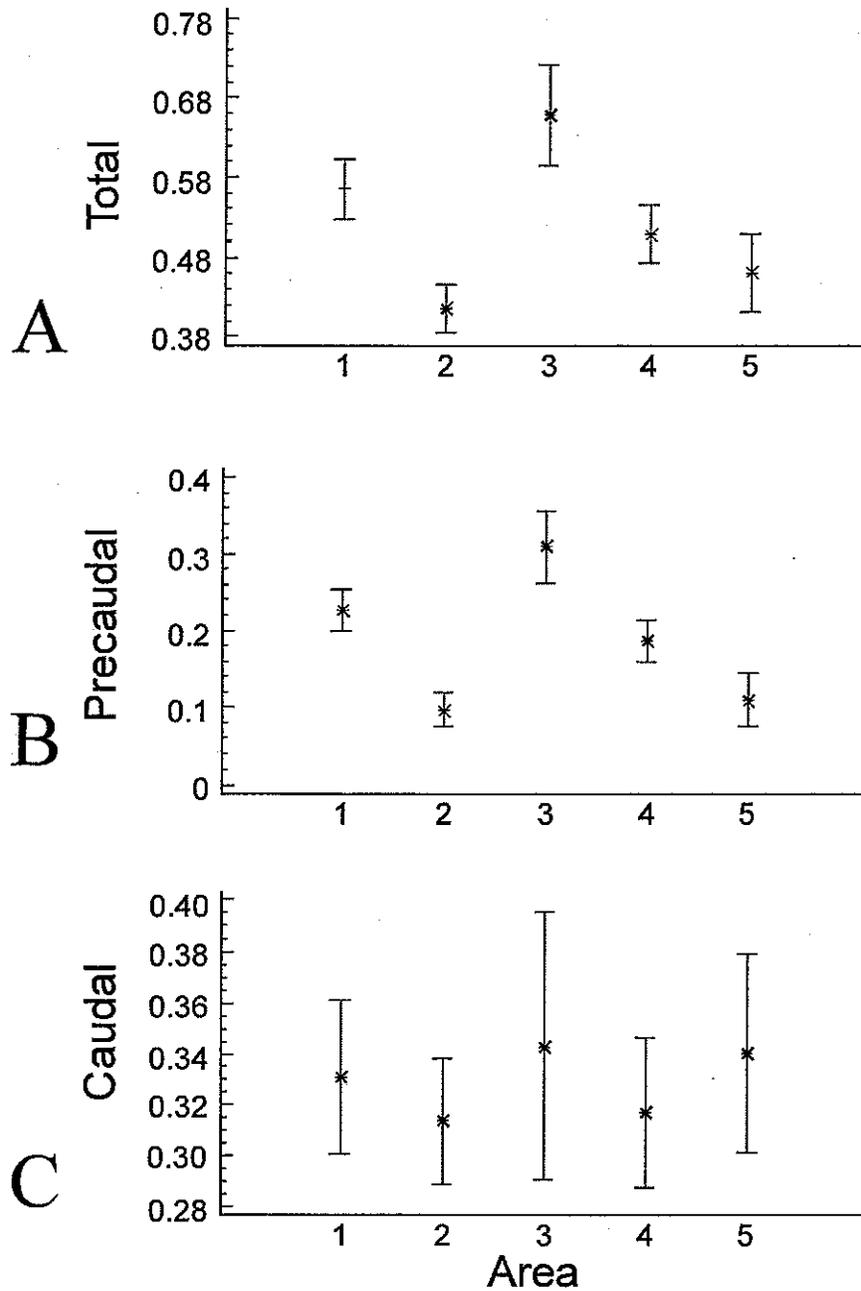


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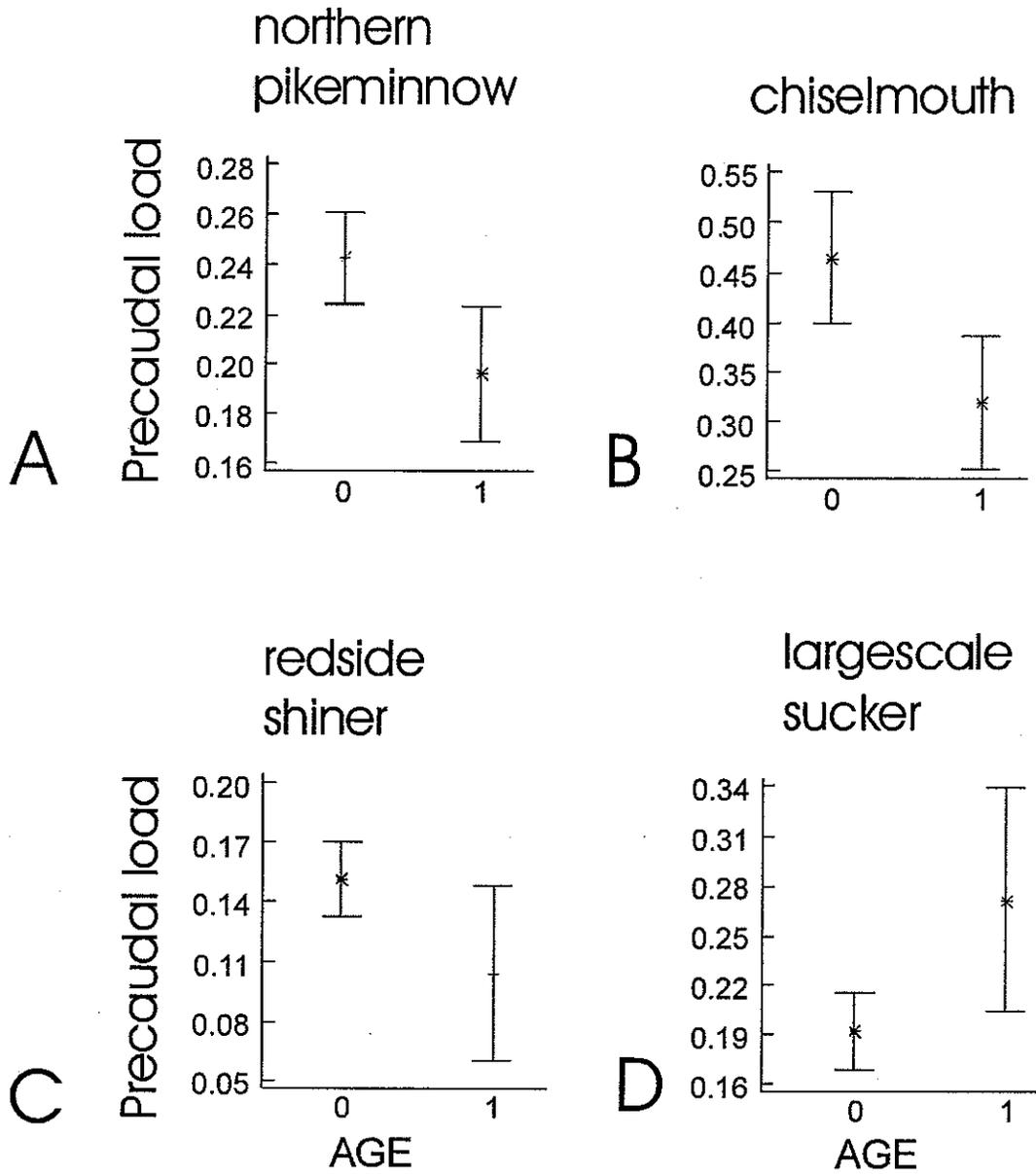


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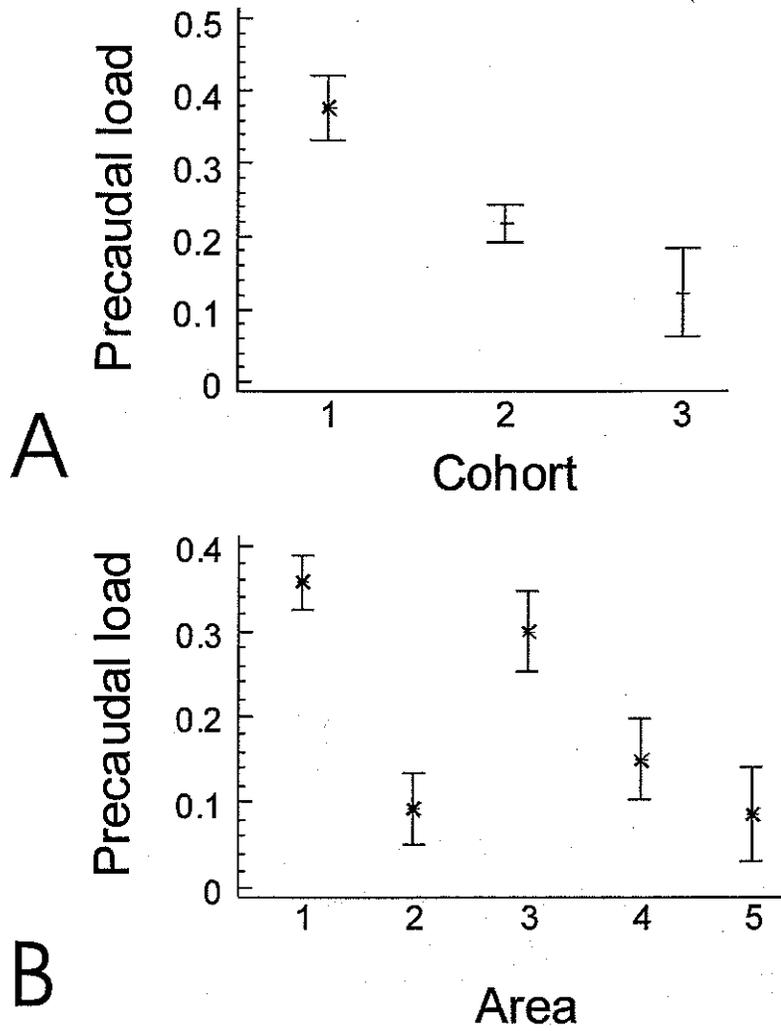


Figure 6. Precaudal deformity load in northern pikeminnow in 2002 and relationships to A) Age 0 cohort where cohort 1 represents earliest and cohort 3 latest hatched fish, N=3720; and B) area, N=5341 including age 1+. Star indicates mean and bars show 99% confidence interval.

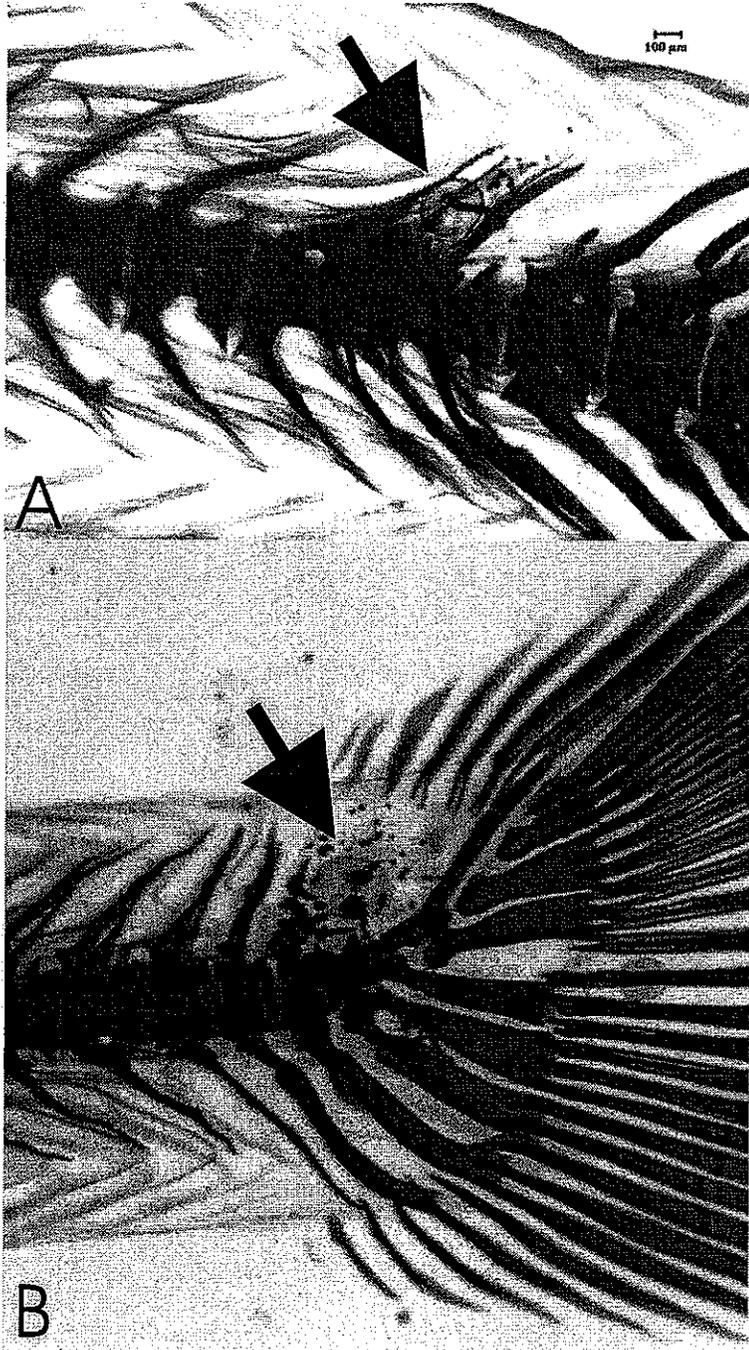


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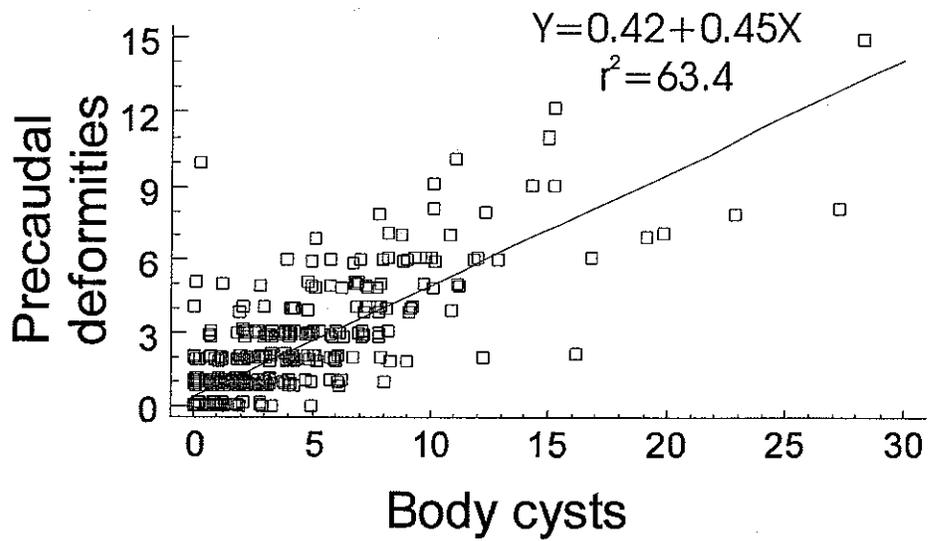


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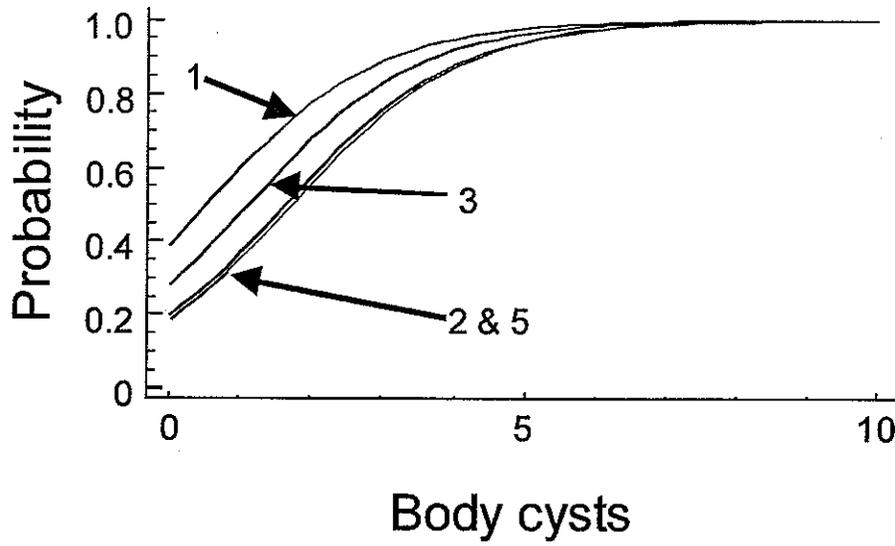


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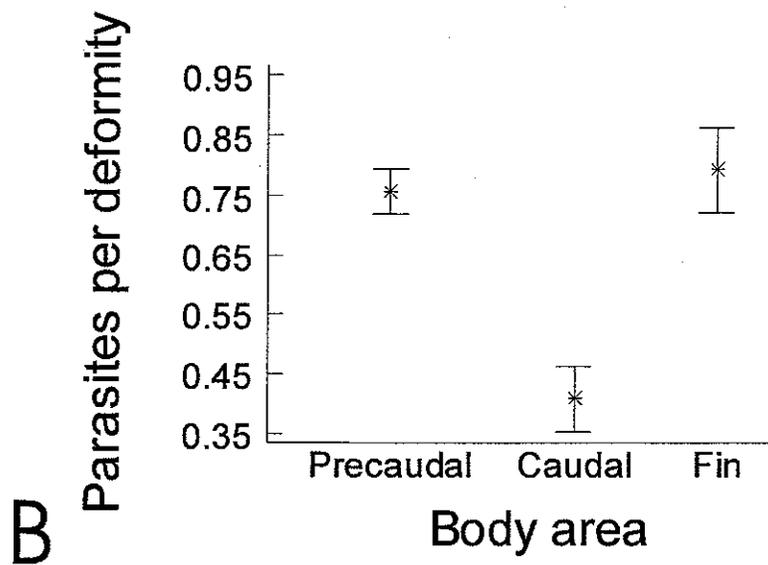
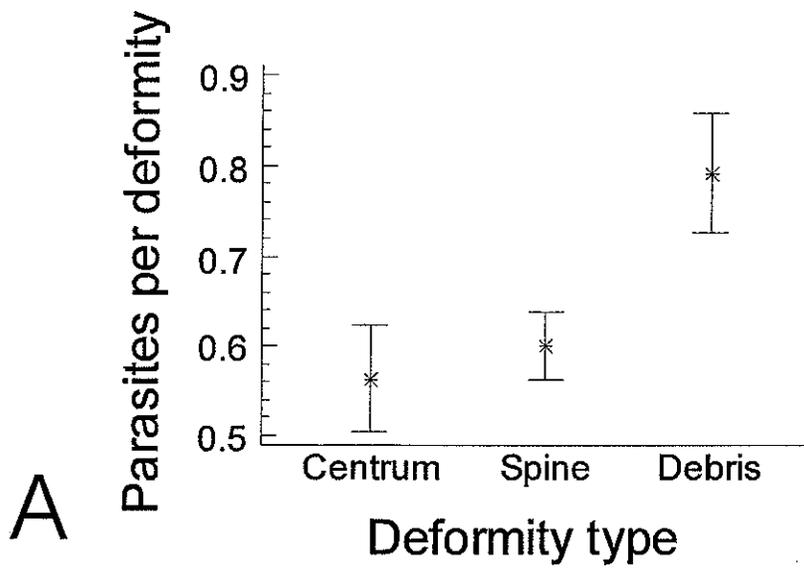


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APPENDIX III

DIGENEAN METACERCARIAE AND A MYXOZOAN
RESSEMBLING *MYXOBOLUS CYPRINI* ASSOCIATED WITH
SKELETAL LESIONS IN CYPRINID FISHES FROM
WILLAMETTE RIVER



Digenean metacercariae and a myxozoan resembling *Myxobolus cyprini* associated with skeletal lesions in cyprinid fishes from Willamette River, Oregon

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Abstract: A high prevalence of skeletal deformities has been observed in various fishes from the Willamette River for many years. Geographic surveys have consistently shown that precaudal skeletal lesions are more prevalent in areas with anthropogenic impact, leading to the hypothesis that toxicant exposure may be the cause. The lesions are common in cyprinid fishes (e.g., northern pike minnow and chiselmouth (*Areochelius alutaceus*). Histological evaluation of 28 underyearling and 20 yearling northern pike minnows *Ptychocheilus oregonensis* showed that about half of the affected fish had infections in which metacercariae of digenean trematodes were directly associated with vertebral lesions. All 33 affected underyearling chiselmouth chub that were examined by histology showed metacercariae associated with vertebral lesions. Metacercariae were also associated with vertebral lesions in three of four affected peamouth chub (*Mylocheilus caurinus*). Many parasites occurred within the vertebral bodies and were associated with melanization and dysplastic and proliferative changes in all three species. In contrast, the parasites were less prevalent in fishes without lesions. We also evaluated the association of the worm with the lesions using cleared fish, a technique which allowed for visualization of encysted worms and skeletal lesions in the same preparation. Fish from the affected area showed a much higher prevalence of metacercariae and lesions, and a higher abundance of worms. Chiselmouth showed more lesions and worms than northern pike minnow. Overall, 79% of lesions were directly associated with metacercariae, and over 94% of the lesions in chiselmouth showed this relationship. Two types of metacercariae were identified in affected fish; *Aphophallus donicus* (family Heterophyidae) based on previous descriptions of this parasite from Oregon fishes and a neascus type (order Strigeidida). The latter were retrieved from fins and muscle, while *Aphophallus* appeared more closely associated with the skeleton based on wet mount preparations of a few encysted parasites. A *Myxobolus* sp. similar to *M. cyprini* (based on spore morphology and ribosomal DNA sequencing) was also associated with the skeletal lesions in 14-15 % of the northern pike minnow, 5% of the chiselmouth, and were not detected in peamouth. Intact plasmodia were found in somatic muscle and inflammatory lesions containing free spores were often localized at bone surfaces. Skeletal lesions occur in very young fish. The myxozoan probably does not play a primary role in the lesions because development of mature spores probably requires several months. This

survey suggests that that metacercariae, and possibly the myxozoan, are probably an important cause of the skeletal deformities seen in cyprinid fishes from certain regions of the Willamette River.

Introduction

Certain regions of the Willamette River in Oregon have been considered to be impacted by anthropogenic contamination, and the Portland Harbor was declared an EPA Superfund site (Waite and Carpenter 2000). A high prevalence of vertebral anomalies in various fish species from this river have been reported by Oregon Department of Environmental Quality (ODEQ). ODEQ studies found northern pike minnow (*Ptychocheilus oregonensis*) in the Willamette River had skeletal anomalies at prevalences of 1 to 74 % with the highest from river mile (RM) 27 to 55, an area known as Newberg Pool (Ellis *et al.* 1997). Prevalence of deformities typically decreases with distance upstream and downstream of Newberg Pool. A subsequent survey by Markle *et al.* (2001) showed no differences in patterns of caudal deformities in Willamette River fishes between river miles 29 and 71, whereas precaudal deformities were substantially higher around river the Newberg Pool area. They reported skeletal lesions in 14 fish species. Most of these fishes were cyprinids, and lesions were most prevalent in chiselmouth chub (*Arcocheilus alutaceus*).

Skeletal deformities in fishes can be caused by a variety of contaminants, including cadmium, lead, zinc, and organophosphate pesticides (Bengtsson, 1974; Bengtsson *et al.* 1975; Bengtsson *et al.*, 1985; Hiraoka *et al.* 1984). Therefore, it has been suggested that skeletal deformities in fish are bioindicators of pollution (Bengtsson 1979; Lemly 1997). However, there are other causes of skeletal deformities that are not directly related to chemicals, including low pH (Frojnar, 1977), elevated temperature (Kwan, 1974), and nutritional deficiencies (Akiyama *et al.*, 1986; Walton *et al.*, 1983; Bengtssen. 1979; Mauck *et al.* 1978; Mehrle *et al.* 1975, 1982). Several infectious agents cause skeletal deformities in fish. These include 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., Bucke and

Andrews 1985; Hedrick *et al.* 1998; Matthews *et al.* 2001; Baturu 1980). A multidisciplinary effort was undertaken by toxicologists and pathologists to

A multidisciplinary effort was undertaken by toxicologists, fisheries biologists, and pathologists to elucidate the cause of these lesions in fishes from the Willamette River, including investigations on potential chemical or infectious causes. Here we report the prevalence, associated pathological changes, and description of parasites from northern pike minnow (*Ptychocheilus oregonensis*) and chiselmouth (*Acrocheilus alutaceus*) from the Willamette River.

Materials and Methods

Underyearling northern pikeminnow, chiselmouth, and peamouth were collected near in the impact zone from Newberg Pool and Wheatland Ferry (River Mile XXX to XXXX) and from our reference site at Kiger Island near Corvallis (river mile XXX), Willamette River from from 20-29 July 2002. Fish were collected by beach seine, and were preserved in 10% buffered formalin. Preserved whole fish in formalin were radiographed with INSTRUMENT and images evaluated for the presence of skeletal deformities as described by (Markle *et al.* 2001).

Preserved fish were decalcified before processing. Tissues were placed in Cal-Ex II solution for 24-48 hrs. Samples were then rinsed in running tap water for 2 hrs, and then returned to 10% buffered formalin for 24 h. Tissues were embedded using standard techniques and sagittal sections were prepared using standard techniques. Larger fish were often processed in pieces. Three sections were prepared from each fish at approximately 50 μ m intervals.

To allow for better correlation with the presence of parasites associated with skeletal changes, fish were also examined 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, T, 1984). Cleared fish were placed in a Petri dish, covered with glycerin, and the entire fish was examined with at 2.5 or 5 X objective. Total numbers of metacercariae and presence and absence of the parasites directly associated with skeletal lesions were recorded. All fish were collected from July 20- 27 2002. Morphological

information from the parasites was obtained using wet mount preparations, and all measurements were made with a SPOT camera and computer program (Sterling Heights, MI).

For molecular analysis of the myxozoan, muscle tissue containing *Myxobolus* sp. spores as determined by microscopy were frozen or placed in 95% ethanol for subsequent molecular analyses. Extraction of DNA employed the QIAgen DNeasy Kit (QIAGEN Inc. Valencia, California). A region of the small subunit (SSU) ribosomal DNA was amplified via the polymerase chain reaction (PCR) using primers, 18E and Myxgen2r, and conditions as described by Kent et al. (2000). Sequence of approximately 850 basepairs was obtained in both directions using the above PCR primers and methods of Whipps et al (2003). To complete the sequence, the 3' region of the SSU rDNA was amplified with primers Mcyp1F (5'-GTCAGTTTGTAGTCTGCG-3') and 18R of Whipps et al (2003). Overlapping sequences were combined, yielding a 1911 bp sequence which was deposited in Genbank (accession # XXXXXXX).

Sequences used for alignment and subsequent phylogenetic analysis were selected based on Genbank searches using the basic local alignment search tool (BLAST) (Altschul et al. 1990) and those used by Molnar et al. (2002). These sequences (with Genbank accession numbers) were as follows; outgroup *Myxobolus lentisuturalis* (AY119688), *Myxobolus bibullatus* (AF378336), *Myxobolus bartai* (AF186835), *Myxobolus pseudodispar* from *Scardinius erythrophthalmus* (AF380142), *Myxobolus pseudodispar* from *Rutilus rutilus* (AF380145), *Myxobolus pseudodispar* from *Abramis brama* (AF380144), *Myxobolus pseudodispar* from *Blicca bjoerkna* (AF380143), *Myxobolus cyprini* (AF380140), *Myxobolus muscoli* (AF380141), *Triactinomyxon* sp. (AY162270). Sequences were aligned with ClustalX (Thompson et al., 1997) and analyzed in PAUP*4.0b1 (Swofford, 1998). Parsimony analysis was conducted using a heuristic search algorithm with 50 random sequence additions. Bootstrap values were calculated with 1000 replicates.

Results

Histology. Metacercariae of digenean trematodes and myxozoan spores were observed in or near bone of vertebrae, spines, and pectoral girdle of many fish for all three host species examined (Fig 1). Metacercariae were observed either within bone or directly appressed to the vertebrae surface in in which the lesion could be located. Parasites were associated with prominent dysplastic and proliferative responses of adjacent bone, often resulting in complete encystment of the parasite (Fig. 1). Metacercariae where also observed directly associated with vertebral spines, where they were either encapsulated with new bone around parasites adjacent to spines or they occurred directly within spines. In both cases, the bony capsule was indistinguishable from bone from normal structures. Examination of radiographs revealed clear regions within dense bone that would correspond to the parasite (Fig. 2).

A histozoic *Myxobolus* sp. was observed in northern pikeminnow and chiselmouth, but not peamouth (Table 1). The myxozoans occurred as intact plasmodia between muscle fibers, often adjacent to vertebral spines. Individual spores were found within a chronic inflammatory matrix adjacent to vertebrae and the spines some fish (Fig. 3). The inflammatory response was comprised of melanin-laden macrophages and associated fibroplasia Both parasites were more prevalent in fish with skeletal lesions than those with out lesions as determined by radiographic analysis (Table 1).

Cleared Whole Mounts. Radiographic analysis provides a three dimensional view of the skeleton, while histology is essentially a two-dimensional technique. We were concerned that several lesions seen by radiography not observed by histology due to difficulties with locating the precise vertebrae with lesions. Therefore, we examined fish in cleared whole mounts in attempt to better determine the association of the metacercariae with the skeletal lesions. Both worms and skeletal lesions were readily visible with this technique, but the myxozoans were not detected.

This method further elucidated the association of trematodes with lesions, which included lordosis, absence of vertebral spines, additional vertebral spines, and bone

proliferation (Figs 4). Multiple, droplet-like, dense, red-staining bodies dystrophic (mineralization of foci of granulomatous inflammation) were frequently associated with the parasite in the surrounding musculature (Fig. 4G). Most skeletal abnormalities evaluated by this method demonstrated metacercariae directly associated with the lesions, particularly in chiselmouth (Table 2). Comparisons between chiselmouth and northern pike minnow revealed that the former had over 7 times as the abundance and about 3 times the prevalence of infection by metacercariae than northern pike minnows in the affected area, and a similar pattern was between these two species in the reference site in Corvallis. Concurrently, chiselmouth showed about twice the prevalence of skeletal lesions compared to northern pikeminnows. Intraspecific comparisons revealed that both species from the affected site had about three times the lesion prevalence, 2-3 times the prevalence of infection by worms, 4-6 times the abundance of worms compared to the control site. Most importantly, skeletal lesions were consistently associated with the worms. This was particularly evident in chiselmouth, which is the most severely parasitized and has the highest prevalence of infection by the metacercariae. For example, over 94 % of the lesions in chiselmouth were associated with worms. Combination of data on lesions associated with worms from both species from both sites revealed that 79% of the lesions were directly associated with visible metacercariae. Direct correlations of overall intensity with numbers of skeletal lesions were not seen as some fish had heavy infections of the fins.

Aggregates of *Myxobolus* spores in the somatic muscle were seen in several northern pikeminnow from affected region, but not in reference site (Table 2). They were not observed in the other samples and spores were not directly associated with lesions.

Parasite Identifications. Wet mount preparations of metacercariae removed from cysts near vertebrae revealed a heterophyid metacercaria consistent with *Aphophallus* sp. (Fig 5A). Distinctive features of the worm identified as *Aphophallus* included spiny tegument, oblique testes, relatively small acetabulum and oral sucker, short prepharynx, small pharynx, and a very long esophagus resulting in division of the caecum in the posterior region of the worm. Strigeid metacercariae (e.g., neascus) with obvious calcareous

corpuscles were found in the muscle and fins within large black, spherical cysts (Fig 5B).

The myxozoan spores observed in muscle tissue of both northern pike minnow and chilsemouth were morphologically consistent with those of the genus *Myxobolus*. Spores most closely resembled those of *M. cyprini*, which has polymorphic spores infecting muscle of cyprinid fishes. Some spores were symmetrical with apical polar capsules, while others have unequal valves, and subapical polar capsules (Fig. 6), and poplar capsules which are subapical. Spores measurements are as follows (n=30, values in μm). Length: 11.0 (10.1-12.6), Width 7.2 (6.7-7.7); large polar capsule length 6.6 (5.5-7.1); small polar capsule 5.3 (4.0-5.9).

We sequenced 1911 bp of the SSU rDNA of the *Myxobolus* sp. from NPM and performed a phylogenetic analysis with other closely related myxozoan sequences available on Genbank (Fig 7). Parsimony analysis yielded two equally parsimonious trees, the only difference between trees was placement of the group containing *M. cyprini* and *M. muscoli*. One tree places these as sister to all *M. pseudodispar* representatives, whereas the other tree places them sister to *M. pseudodispar* from rudd (*Scardinius erythrophthalmus*) and roach (*Rutilus rutilus*). Regardless, the *Myxobolus* species from NPM is an outlier to all of these species with strong bootstrap support (Fig. 7).

Discussion

The cause of the high prevalence of skeletal deformities seen in fishes from the Willamette River near Portland has been suspected to be an unknown contaminant for many years. Using multiple diagnostic methods, survey of fish collected from the affected and reference sites suggest that that most of these lesions are caused by parasites. The association with heterophyid metacercariae is most compelling from both the diagnostic and epidemiological aspects. Baturu (1980) induced skeletal changes in cyprinids by exposing them to cercariae of *Bucephalus polymorphos*. Metacercariae of heterophyid digeneneans that infect the gills of freshwater fishes have been reported to cause prominent dysplastic and proliferative changes in the gill cartilage (Blazer and Grazek 1985; Olson and Pierce 1997). The parasite in the present study was identified as an *Apophallus* species based on morphology of the metacercariae. Niemi and Macy (1974) described the life cycle of an *Apophallus* from fishes collected in the Willamette River basin Oregon. As in the present study, second intermediate hosts included northern pike minnow, as well as various other fishes. Moreover, they experimentally infected coho salmon (*Oncorhynchus kisutch*) in which the metacercariae were found in bony structures. Neimi and Macy (1974) proposed that *A. venustus*, *A. similes*, and *A. brevis* were synonyms of *A. donicus*. Review of the taxonomy of the genus is warranted, but we can conclude that the *Apophallus* sp. in the present study was likely *A. donicus* as described by Neimi and Macy (1974). These authors reported little host specificity for the metacercariae of *A. donicus* in Oregon, which is might explain why lesions have found in many fish families (Markle et al. 2001).

Taylor et al (1993, 1994) described the induction on ectopic bone associated with infection by *A. brevis* in yellow perch (*Perca flavescens*). In the present study, the metacercariae occurred directly within the body of the vertebrae or immediately at the surface of the bone. In both cases, the encysted parasites caused prominent chronic inflammation, and dysplastic and proliferative changes of the bone in the vertebrae. These changes certainly would be reflected in anomalies visualized by radiographic analysis. Indeed, retrospective examination of deformities seen in radiographs revealed

dense regions (corresponding to regions of bone proliferation) and clear centers (corresponding to parasite cyst) (Fig 2). Such vertebrae often were fused and had additional spines, and common deformity reported by Markle et al. (2001).

Examination of cleared fish added to our understanding of the trematode association and pathogenesis of the lesions. A stronger association of the worm with the lesions was seen by this method, probably because we were able to detect worms and skeletal lesions in the same preparation. In other words, detection of skeletal lesions in cleared fish was more reliable than in histological sections. Indeed, most of the vertebral lesions were associated with metacercariae. Most lesions without worms were characterized by increased density and fusion of centra using the clearing method (Fig 4). Worms or their degenerated remains may have gone undetected deep within these structures as they were detected deep within vertebrae by histology (Fig 1A). Cleared preparations showed bone directly associated with the parasite, the direct location of the worms in the region with lordosis was clearly demonstrated. Several worms were associated with mineralized droplet-like structures in the surrounding muscle. This was most likely dystrophic mineralization of foci of granulomatous inflammation. One parasite that was completely encased in new bone exhibited a pore corresponding to the ossicle canal described for *A. brevis* in yellow perch (Taylor et al. 1994). Direct correlations with overall body intensity with numbers of skeletal lesions were not seen as a few fish had heavy infections of the fins. We could not differentiate between neascus and *Apophallus* in cleared fish, and thus it is possible that many of the fin infections were the neascus rather than *Apophallus*. Chiselmouth from the affected region showed 10 times worm abundance compared to those from the reference site, and 2.5 times as many lesions. Chiselmouth also were more heavily infected than northern pike minnow. Markle et al. (2001) and the present study found fewer lesions in this species compared to chiselmouth. These observations provide further support that the worm is a primary cause of the deformities.

To our knowledge, this is the first report of *Apophallus* sp. eliciting proliferation of existing bone. Based on histology and cleared fish, it appears that some worms either actually penetrate vertebrae or worms adjacent to skeletal structures induce proliferation of bony material around the parasite. In contrast, *Apophallus brevis* metacercariae infects the somatic muscle of yellow perch (*Perca flavescens*) muscle and causes a host response comprised of ectopic bone (Sinclair 1972; Pike and Burt 1983; Taylor et al 1994). As in

the present study, *A. brevis* in yellow perch does not apparently infect the visceral organs (Pike and Burt 1983). Other infections by other metacercariae have been linked to skeletal anomalies. Muscle infections by *Bucephalus polymorphos* causes skeletal deformities in cyprinid fishes (Baturu 1980), and *Riberiora* sp. has emerged is now suspected to be a major cause of supernumerary limbs and other skeletal changes in seen in frogs in North America (Kaiser 1999).

Myxozoans, particularly *Myxobolus* spp., that infect bone are recognized causes of skeletal deformities. In addition to the *Myxobolus cerebralis* of salmonids (see reviews by Hedrick et al. 1998 and Bartholomew and Reno 2002), vertebral anomalies are associated with *Myxobolus sandrae* infections of yellow perch (*Perca fluviatilis*) in Scotland (Treasurer 1992; Lom et al. 1991), *M. ellipsoides* in European chub (*Leuciscus cephalus*) in England (Bucke and Andrews 1985), *M. cartilaginis* in centrarchid fishes in the United States, and *Triangula percae* (Myxozoa) in yellow perch in Australia (Langdon 1987).

The parasite in our study was most similar to *Myxobolus cyprini* or *M. pseudodispar* in spore morphology, site of development, and host. Spores of all three infect the muscle of cyprinid fishes and have distinctly pleomorphic spores. Some authors have suggested that *M. pseudodispar* is a junior synonym of *M. cyprini* (Lom et al 1992). In contrast, Molnár et al. (2002) proposed that *M. cyprini*, *M. pseudodispar* and *M. musucli* should be maintained as separate species based on rDNA analyses (although the former 2 are morphological indistinguishable). Free spores of *M. cyprini* from ruptured plasmodia are disseminated to various organs where they induced prominent inflammatory changes (Molnár and Kovács-Gayer. 1985). Kent et al. (1996) was the first report of the parasite in North America. In contrast to previous reports, we observed free spores accumulated near skeletal structures and we did not detect them in visceral organs. Moreover, our molecular analysis showed that it 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 study to these species because it would render the species paraphyletic.

The myxozoan was more common in northern pike minnow than chiselmouth and was not seen in peamouth by histology. Moreover, fish with the myxozoan were more

often seen in fish with lesions using this method. In cleared fish, the myxozoan was only seen in northern pikeminnow from Newberg Pool. Only large aggregates of spores were seen in cleared fish and they were not directly associated lesions. This is consistent with histological observations in that only individual spores within macrophages were seen in lesions, and it is doubtful that we would have seen these spores in cleared fish. Two possibilities exist regarding the association of the *Myxobolus* sp. with the skeletal lesions; either the myxozoans cause some of the vertebral lesions or the free spores migrate to preexisting lesions which are replete with macrophages. Evidence supporting the latter includes the fact that the skeletal lesions are observed in fish as young as 30-60 d, while spores of *M. pseudodispar* in experimentally infected fish require at least 80 d to develop (Székely et al. 2001). Additional time would be required for plasmodia to enlarge, rupture, and release spores. Spores of *M. cyprini* accumulate in regions of the host that are replete with macrophages (Molnár and Kovács-Gayer. 1985). In the present study, spores accumulated in inflammatory lesions around vertebrae rather than in the kidney and spleen. It is possible that these lesions may represent sites where the worm had caused the initial lesion and then was destroyed by the host or the worm was not seen in the histological section. The latter option is likely because most lesions were associated with worms when evaluated in cleared fish preparations (Table 2). Nevertheless, the *Myxobolus* sp. in cyprinids from the Willamette River are probably a contributing factor to the severity and prevalence of the skeletal lesions.

In conclusion, evidence presented here strongly indicates that the metacercariae is a digenean trematode (probably the *Apophallus* sp.) and plays a significant role in the high prevalence of skeletal lesions in the Newberg Pool region of the Willamette River. Further support of this hypothesis is that the lesions have been seen in fish collected as early as 1952 (Markle et al. 2002) and our investigations on potential chemical contaminants in these fish and in water from Newberg Pool have not revealed any likely candidates (Curtis, L. pers. comm.). Other questions remain, such as the apparent relationship on the infection and skeletal lesions with urbanized regions of the river. Increased trematode infections have been related to human activities (Lardans and Dissous 1998), and Kiesecker (2002) reported a synergism between exposure to herbicides and pesticides and infections by metacercariae of *Ribeiroia* sp. and *Telochris*

sp. relating to observations of skeletal deformities in frogs. While we have not detected contaminate levels that would suspect of causing the lesions, it is possible that an unknown agent occurs in the affected region that causes immunosuppression of cyprinid fishes in the river and thus predisposes the fish to infection. Observation of increased prevalence of two unrelated parasites in the Newberg Pool region fish supports this hypothesis. However, it is also possible that factors not directly related to pollution, such as water flow dynamics, abundance of non-fish hosts involved in the life cycles of *Myxobolus* and *Apophallus*, are important contributing factors to the higher parasite burden in the Newberg Pool region. We plan to conduct studies with laboratory-reared fish experimentally exposed to cercariae from the snail host for *Apophallus* in Oregon (*Flumenicola virens*) to more precisely determine the association of this parasite to the lesions. Furthermore, more precise identification the species of the *Apophallus* in the present study requires experimental infections with appropriate definitive hosts and review of previous descriptions of members of this genus. Another survey has been initiated to include a much larger sample size and multiple year collections to further elucidate the potential links of the parasites to the deformities.

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Table 1. Histological evaluation of prevalence and association of parasites with lesions compared with skeletal deformities identified by X-ray analysis in chiselmouth (CHIL) northern pike minnows (NPM), and peamout (PEAM) collected June-August 2002 from Newberg Pool, Willamette River. Chiselmouth were identified as underyearlings, peamouth as yearlings, and NPM where divided into 2 groups; -1 = underyearlings and +1 = > 1 year old. "Lesions" indicates parasites associated with vertebral lesions. Note the denominator of "Lesions" is often smaller than "Prevalence" as lesions were often not seen in sections.

Fish	X-ray	Metacercariae		<i>Myxobolus</i>		
		No.	Prevalence	Lesions	Prevalence	Lesions
NPM(-1)	+	28	16/28 (57)*	11/27 (41)	10/28 (36)	4/27 (15)
NPM(-1)	-	18	4/18 (22)	NA†	3/18 (17)	NA
NPM(+1)	+	20	11/20 (55)	8/14 (57)	18/20 (90)	11/14
NPM(+1)	-	4	1/4 (25)	NA	3/4 (75)	NA
CHIL	+	33	33/33 (100)	21/21 (100)	6/33 (18)	1/21 (5)
CHIL	-	8	8/8 (100)	NA	3/8	NA
PEAM	+	4	3/4 (75)	3/4 (75)	0	0
PEAM	-	4	1/4	NA	0	NA

* Numbers infected/numbers examined (percent prevalence)

† Not applicable as lesions were not observed in these fish

Table 2. Association of metacercariae with skeletal lesions in northern pikeminnow (NPM) and chiselmouth (CHM) from Newberg Pool region (near Portland) and Kiger Island (Corvallis), Willamette River collected 21 – 29 July 2002. Based on microscopic examination of whole, cleared fish stained with alcian blue and alizarin red S.

Species	Size Mean (Range)	No.	Location	Lesions		Infection		Mean		Lesions with worms		<i>Myxobolus</i> Prevalence (%)
				Prevalence (%)	Prevalence (%)	Prevalence (%)	Prevalence (%)	Abundance	(%)	(%)		
CHM	3.1 (2.6-3.8)	37	Affected	64.5	94.5	4.62	94.7	0				
CHM	2.2(1.9-2.4)	14	Reference	21.4	43	0.92	100					
NPM	2.8 (1.9-4.5)	37	Affected	35.0	38	0.62	54	19				
NPM	2.2 (1.7-2.7)	18	Reference	11.1	11.1	0.10	50	0				



FIGURES

Figure 1. Histological sections of metacercariae in northern pike minnow. H&E. Bar = 100µm. A. Encysted metacercariae in vertebra associated with bone and cartilage proliferation. B. Metacercaria associated with deformed dorsal spine and chronic inflammation. C. Bony cyst in neural spine at base of vertebrae. D. Bony cysts in spine with melanized host reaction.

Figure 2. Radiograph of vertebral lesion. Arrow indicates region suggestive of worm infection; dense region represents bone proliferation with lucent center corresponding to encysted worm.

Figure 3. *Myxobolus cyprini* in histological sections of northern pikeminnow with skeletal deformities. A. Intact pseudoplasmodium next to dorsal vertebral spine. B. C. Low magnifications showing inflammatory lesions associated with skeletal deformities. Box indicates region shown in high magnification in Figure 3E. D, E. High magnification showing spores (arrows) in chronic inflammation.

Figure 4. Metacercariae in cleared northern pike minnow (A-D) and chiselmouth (E-G). Bar = 200 µm. A. Metacercaria at a site of missing hemal spine. B. Dense vertebrae (arrow), possibly associated with internal parasite (see Fig 1A). C. Bony proliferation partially encasing metacercaria at base of hemal spine. D. High magnification of C. E. Bent spine with associated metacercariae. F. Dystrophic hemal spines associated with two metacercariae. G. Additional neural spines and multiple dense concretions (arrows). H. Metacercaricae cyst encased in bony material at base of neural spine. Note stock-like structure (arrow) connecting cyst to veterbrae and pores in cyst.

Figure 5. Wet mount preparations of metacercariae from northern pike minnow. Bar = 100 µm. A. *Apophallus* sp. B. Strigeid (neascus) with calcareous corpuscles (arrows) within melanized cyst.

**Figure 6. A. Spores of *Myxobolus cyprini* in wet mount preparations. Bar = 10 μm .
B. Aggregates of spores in somatic muscle of cleared northern pikeminnow. Bar = 100 μm**

Figure 7. Phylogenetic analysis of *Myxobolus* sp. from northern pike minnow.

RAW DATA for Table 2

Fish Number	Species Location	Number of Skeletal Lesions	Total Number Metacercariae	Number of Lesions with Metacercariae	Size (cm)
118		1	1	1	3.2
117		1	7	1	3.5
116		1 (dense)	6	0/1	3.4
115		1	17	1	3.7
114		0	9 (all in tail)	0	3.7
113*		2	3	2	3.3 photo
112		0	1	0	
111		0	4	0	3.8
110		2	5	2	3.5
109		1	4	1	3.7
108		1	2	1	3.2
107		1 (dense vert.)	4	0/1	3.6
106		0	1 (melanized)	0	3.2
105		1	11	1	3.8
104		0	4	0	3.8
103	Ch/New	0	1	0	3.7
82		0	0	0	2.2
81		1	6	1	2.5
80		1	2	1	1.9
79		2	6	2	2.2
78		0	2	0	2.8

77		0	14	0	2,7
76		3	6	3	2.7
75	Extras spine, drops , worm in spine near tail	2	2	2	2.9
74		2	8	2	3.0
73		1 bent spine	1	1	2.6
72		0	4	0	2.6
71		1 vert dense, v15-17	4	1	2.8
70		4	7	2	3.4
69		3	10	3	2.8
		V6,7 droplets. V11-13 twisted neural spines and droplets			
68		2	3	2	3.1
		V23 & 31, both extra spines			
67		0	2	0	2.8
66		0	1(v 10)	0	2.8
65		2	2 (v22,42	2	2.7
64		2	7	2	3.2, bent spine with worm PHOTO
63		0	0	0	2.3
62-46					
NPM,					
Kiger					
62		1 V3 from tail	0	0	1.7

1. Prevalence of lesions based on X-rays. Consistent with previous studies on fishes from the Willamette River, skeletal lesions based on X-ray evaluations were more prevalent in the Newberg Pool region south of Portland compared to our reference site at Corvallis (Table 1 **Mike/Doug – include overall results from 2002?? We could or just say ‘unpublished’.** It depends on direction manuscript takes and whether information adds to point(s) of paper. A subset of fish fish from both the Newberg Pool and Corvallis site were examined by histology (Table 2). This included both underyearling (< ??? cm) and 1-2 year-old-fish. How was subset chosen – random, “representative”, haphazard? I think we have some insight into the fish-end of the biology (below) that has an impact on how you interpret the subsets.

Fish anomalies and parasite loads in agricultural areas of the basin were noted to be high (Wentz et al 1998; Waite and Carpenter 2000),

Typically, fish populations from non-impacted should have skeletal deformity rates of 2 to 5 % (Gill and Fisk 1966; Wells and Cowan 1982). Newberg Pool is a rather slow, deep section of the lower river immediately upstream of Willamette Falls
Cleared Fish Data