AN ABSTRACT OF THE THESIS OF

Jon M. Harden for the degree of Master of Science in Toxicology presented on September 23, 2003.

Title: Developmental Axial Skeletal Deformities: Baseline Study with a Zebrafish Model.

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Abstract approved

Lawrence R. Curtis

The aim of this study was to assess sensitivity of early life stage zebrafish to cadmium (Cd). Embryos and larva were exposed to Cd before the formation of skeletal elements. Exposure times were selected to determine whether Cd perturbed developmental processes that lead to skeletal deformities, and to characterize the types of skeletal deformities that occurred. Embryo/larva were exposed to 3μM, 10μM, 30μM, 100μM, 300μM, and 1000μM Cd at 12-36 hours post fertilization (hpf), 36-60hpf, 60-84hpf, and 144-168hpf in one series of experiments. This experiment was conducted at circumneutral pH. A second series of experiments with these same Cd concentrations were also conducted at pH 6 with embryo/larva exposed at 12-36hpf, 36-60hpf, and 60-84hpf. The fish that survived the exposures were raised for 40-60 days whereupon the surviving fish were overdosed with MS222, and X-rayed. The later early life stage fish were more sensitive to Cd toxicity; the chorion appeared to protect the earlier exposed embryos (zebrafish hatch 48-72hpf). Embryo/larva were more resistant to toxicity from dissolved Cd (pH 6) than particulate Cd (circumneutral pH); absence of functional gills during early life stages perhaps explained resistance to dissolved Cd. Notochord lesions (typically lethal within two weeks) occurred when embryos were exposed 12-36hpf. There was no evidence for sensitivity of early life stage zebrafish to Cd induced skeletal deformities that occurred within 40-60 days of Cd exposures.

Developmental Axial Skeletal Deformities: Baseline Study with a Zebrafish Model.

By
Jon M. Harden

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APPROVED:

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Major Professor, representing Toxicology

Redacted for Privacy

Head of the Department of Environmental and Molecular Toxicology

Redacted for Privacy

Dean of the Graduate School

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Developmental Axial Skeletal Deformities: Baseline Study with a Zebrafish Model

Chapter 1
Introduction

Skeletal deformities in fish may be good bioindicators of pollution, and how the deformities are patterned could indicate chronic or acute exposures. Chronic exposure may be indicated when the frequency of anomalies increases with age of fish, and acute exposure if deformities are found in early life stages. Though the mechanisms are not often fully understood, some scientists suspect developmental processes are disrupted. If skeletal deformities are used as an environmental monitor, it should be recognized that multiple processes may be contributing. For example, factors contributing to skeletal deformities in the head may not affect the formation of the caudal skeleton. Markle et al. (2002) showed that, in 2000, patterns of caudal deformities in Willamette River fishes showed no differences between river miles 29 and 71 while precaudal deformities were substantially higher around river miles 45-50, the Newberg Pool area. Thus, it is important to partition skeletal deformities into different categories, some of which, caudal deformities, may indicate low-level or no interference with developmental process, and some of which, precaudal deformities, may indicate severe interference (Markle et al., 2002).

A literature review revealed a large number of environmental contaminants and natural factors produced skeletal deformities in fish: heavy metals, pesticides, temperature, nutrition, genetics, and parasites were examples. At the start of this study heat and cold shock, retinoic acid (effects expression of HOX genes), the insecticide chlorpyrifos (a cholinesterase inhibiter), and cadmium (Cd) were assessed as potential

means to induce skeletal deformities in early life stage exposures of zebrafish. In preliminary studies Cd induced notochord lesions (i.e. a bent and twisted notochord), and was selected for further study.

Cadmium is considered to be carcinogenic, teratogenic (Klaasson, 1996; Marquardt et al., 1999), and to cause skeletal deformities in fish (Bengtsson, 1975, 1979, 1985; Hiraoka and Okuda, 1983, 1984). Cadmium induces ionic imbalances (Larsson, 1975), and probably affects the neural muscular system of adult fish by prolonging muscle action potentials, and the ensuing contractions fracture the vertebrae (Bengtsson, 1975, 1979, 1985; Larsson, 1975). Other possible mechanisms of Cd toxicity could be a strong affinity for sufhydryl groups (Bodek et al., 1988; Marquardt et al., 1999), or competition with other divalent ions (Ca^{++}, Mg^{++}, or Zn^{++}) for protein binding sites (Bodek et al., 1988; Chow and Cheng, 2003).

Early life stage studies of exposures to Cd are numerous (Cheng et al., 2000; Chow and Cheng, 2003; Hiraoka and Okuda, 1983, 1984; Williams and Holdway, 1999; Witeska et al., 1995; Woodworth and Pascoe, 1981). Williams and Holdway (1999) examined 2 hour pulsed Cd exposures on spotted rainbow fish (*Melanotaenia fluviatilis)*, and Woodworth and Pascoe (1981) exposed rainbow trout (*Oncorhynchus mykiss)* to Cd continuously from fertilized eggs to swim up; both of these studies report spinal flexure in the larva, but neither raised the fish to adulthood. Hiraoka and Okuda (1983) found that medaka (*Oryzias latipes)* exposed continuously to 0.01ppm and 0.001ppm Cd from eggs to adult had significantly more deformed centra than the controls. The number of deformed centra that occurred in adult medaka exposed to 0.01ppm Cd from fertilized eggs until the retinal pigmentation stage was not

statistically different from the control. Hiraoka and Okuda (1984) again studied the number of deformed centra that occurred in adult medaka exposed to 0.01ppm Cd from fertilized eggs until the retinal pigmentation stage, and it was statistically different from the control ($P<0.01$, χ^2-test). Cheng et al. (2000) examined the "Cellular and molecular basis of cadmium-induced deformities in zebrafish embryos" and found: lack of expression of sonic hedgehog gene, which controls patterning of somites, is associated with head and eye hypoplasia; ecotopic expression of the genes *evenskipped 1* and *no tail* in embryos with tail malformations; and reduction of somite area and myotome formation where axial curvature occurred.

The axial skeleton of fish develops at early life stages. In zebrafish from 10-24 hpf, the precursors of the axial skeleton, the notochord and somites, form in a cephalic to caudal progression. During this segmentation period the somites differentiate into myotomes (muscle segments) and sclerotomes (which give rise to the vertebral cartilage). Chondrogenisis, the formation of cartilage, begins 48hpf, and the formation of the first bone, the cleithrum, occurs 72hpf (Kimmel et al., 1995). The 3 anterior most vertebrae begin to form at 6-7 days post-fertilization and all vertebral centra are present after 9 days. Development of ribs, neural arches and haemal arches is complete 21 days post-fertilization (Moren-Kensicki et al., 2002).

The aims of this study are: to develop a positive control for axial skeletal deformities to be used in future bioassays, to determine when in the developmental process Cd can induce skeletal deformities, and to characterize what type of skeletal deformities occur. The time periods for exposure correspond with somitogenisis, chondrogenisis, and the ossification of the first 3 anterior vertebrae. Axial skeletal

deformities are characterized according to increasing severity: caudal < precaudal < caudal/precaudal < spinal curvature. The null hypotheses tested are: There is no temporal difference in mortality; there is no temporal difference in the occurrence of skeletal deformities; there is no temporal difference in the types of skeletal deformities occurring.

Chapter 2
Materials and Methods

Embryos of the zebrafish *Danio (Brachyodanio) rerio* TL line were obtained by natural spawnings from the zebrafish colony at Oregon State University's (OSU's) Marine and Freshwater Biomedical Science Center. The embryos were maintained at 27°C with a 14 hour light: 10 hour dark photoperiod, and staged according to established criteria (Kimmel et al., 1995). To prevent overcrowding embryos were raised in 2L Rubbermaid containers (15-20 per container). Embryo solution (0.14M NaCl, 5.4mM KCl, 0.25mM Na_2HPO_4, 0.44mM KH_2PO_4, 1.3mM $CaCl_2$, 1.0 mM $MgSO_4$, 4.2mM $NaHCO_3$) used charcoal filtered, UV irradiated well water (Westerfield, 2000). Larva were fed Microfeast L-10 Larval Diet (Burns Philip Food Inc.) and Hatchfry Encapsulon (Argent Chemical Laboratories) from 5-12 days post fertilization (dpf), and Argentemia® Brine Shrimp (Argent Chemical Laboratories) from 10 dpf through grow out.

A preliminary experiment included six water-born nominal cadmium chloride (Sigma Chemicals) concentrations (0, 10μM, 30μM, 100μM, 300μM, and 1000μM). Embryo/larva were exposed at 12-36hpf, 36-60hpf, and 60-84hpf (conducted at circumneutral pH). For each exposure period, modified 24 well inserts containing embryo/larva (n=48) were placed in trays containing one of the six cadmium concentrations. A dissecting microscope (Wild Makroskop M420) was used, for the first time period, to pipette fertilized eggs (staged from 70% epiboloy to 2 somites) that were placed in containers; fertilized eggs for the remaining time periods were visually pipetted and placed in containers at 34hpf. Embryo/larva were raised in

24 well culture plates. Animals were raised until 21dpf and overdosed with MS222 (200mg/L); mortality and abnormalities were monitored daily, and photographed (Wild Photoautomat MPS 45; Kodak Select Series film, 35mm 160T).

Subsequent concentration response experiments included seven water-born cadmium chloride concentrations (0, 3μM, 10μM, 30μM, 100μM, 300μM, and 1000μM). Embryo/larva were exposed 12-36hpf, 36-60hpf, 60-84hpf, and 144-168hpf at circumneutral pH (Experiment I), or 12-36hpf, 36-60hpf and 60-84hpf at pH 6 (Experiment II). For each exposure period plastic inserts containing embryo/larva (n=20, Experiment I; n=15, Experiment II) were placed in beakers containing one of the seven cadmium concentrations. The cadmium concentrations were verified at OSU's Crop and Soil Science Central Analytical Laboratory by inductively coupled plasma spectrometry. A dissecting microscope was used, for the first time period, to pipette fertilized eggs (staged from 70% epiboloy to 2 somites) that were placed in containers; fertilized eggs for the remaining time periods were visually pipetted and placed in containers at 34hpf. In Experiment I mortalities were recorded at 96hpf, and in Experiment II mortalities were recorded 10dpf. Animals were raised to 40dpf in Experiment I, 60dpf in Experiment II, overdosed on MS222, and X-rayed (Faxtron model MX 20) with Structurix D4 DW ETE X-ray film (AGFA-Gevaert N.W.) set for 180 minutes at 18-19Kv.

SYSTAT version 9.0 for windows was used for statistical analysis. A modified arsine transformation ($1/2[\text{arsine } \sqrt{(x/n+1)} + \text{arsine } \sqrt{(x+1/n+1)}]$) was used on percent data (Zar, 1999). One and two-way ANOVA were used to analyze total survival, and

axial skeletal deformities ($\alpha = 0.05$). Pair-wise comparisons were made with Bonferoni post hoc test. Binary logistic regression was used to calculate LC50's.

Chapter 3
Results

A preliminary Experiment was conducted at circumneutral pH. Notochord lesions occurred after 12-36hpf exposures (Fig. 1). The lesions were typically lethal 7-21dpf. Mortalities 7 days post exposure (dpe) were 100% for 1000μM Cd at 36-60hpf and 60-84hpf (Table 1). There were high rates of mortalities (overall average of 94%) for all groups in the preliminary experiment. Handling was reduced in subsequent studies.

Experiment I was conducted at circumneutral pH, and significant precipitation was apparent. Analysis by inductively coupled plasma spectrometry confirmed the precipitate contained Cd (data not shown). Table 2 shows the results of a two-way ANOVA analysis of mortality (40dpf) vs. nominal Cd concentrations ($F=18.825$, $P<0.001$). The mean mortality for 1000μM of Cd is significantly different than for other concentrations ($P<0.001$). There was a significant interaction between the times of exposure and the concentrations used ($F=2.899$, $P=0.003$). Figure 2 shows that 1000μM of Cd produced different mean mortality for exposures at 36-60hpf and 60-84hpf than other Cd concentrations ($P<0.007$ and $P<0.003$ respectively). For the first three exposure periods (40dpf) 1000mM of Cd produced 65%, 98%, and 99% total mortality respectively (Table 3). Exposure at 144-168hpf produced 100% mortality for concentrations of 30μM of Cd and higher.

Two-way ANOVA (Table 4) showed a significant difference between the types of skeletal deformities that occurred in fish 40dpf ($F=49.899$, $P<0.001$). Bonferoni pair-wise comparison (FIG. 3) showed that the mean for caudal

Figure 1: Notochord lesion that developed after embryos were exposed to 100uM of cadmium 12-36hpf.

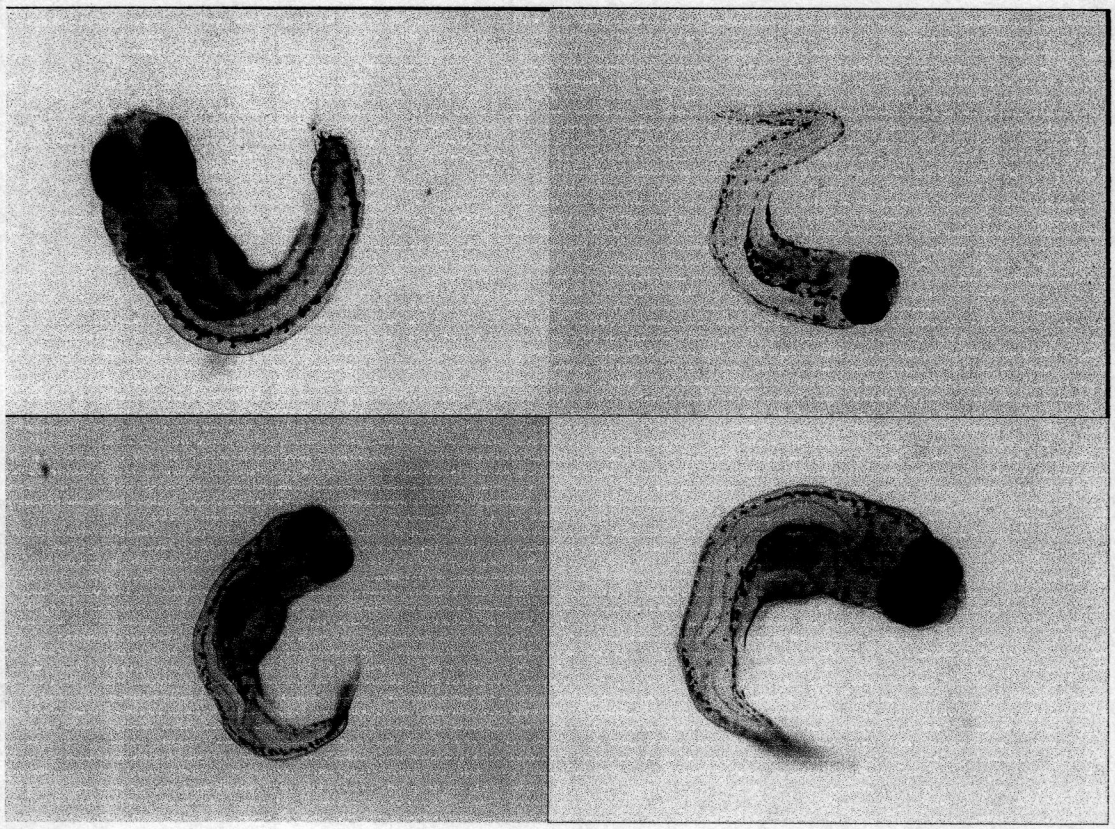

Table 1: Percent mortality of zebrafish larva 7 days post exposure from the Preliminary Experiment of abnormalities (Appendix 1).

	Control	**10μm**	**30μM**	**100μM**	**300μM**	**1000μM**
12-36hpf	20.8%	27.1%	16.7%	12.5%	25.0%	52.1%
36-60hpf	20.8%	22.9%	27.1%	54.2%	20.8%	100%
60-84hpf	20.8%	64.6%	45.8%	95.8%	79.2%	100%

Table 2: Results from a two-way ANOVA (Experiment I) on mortality (α=0.05). There are three levels of time (12-36hpf, 36-60hpf, and 60-84hpf), and seven levels of concentration (0, 3μM, 10μM, 30uM, 100μM, 300μM, and 1000μM).

	F-ratio	**P-value**
TIME	1.221	=0.302
CONC	18.825	<0.001
TIME*CONC	2.899	=0.003

Figure 2: Total percent mortality in Experiment I. The star denotes $P<0.05$, and error bars use standard error. Concentrations used are nominal.[A]

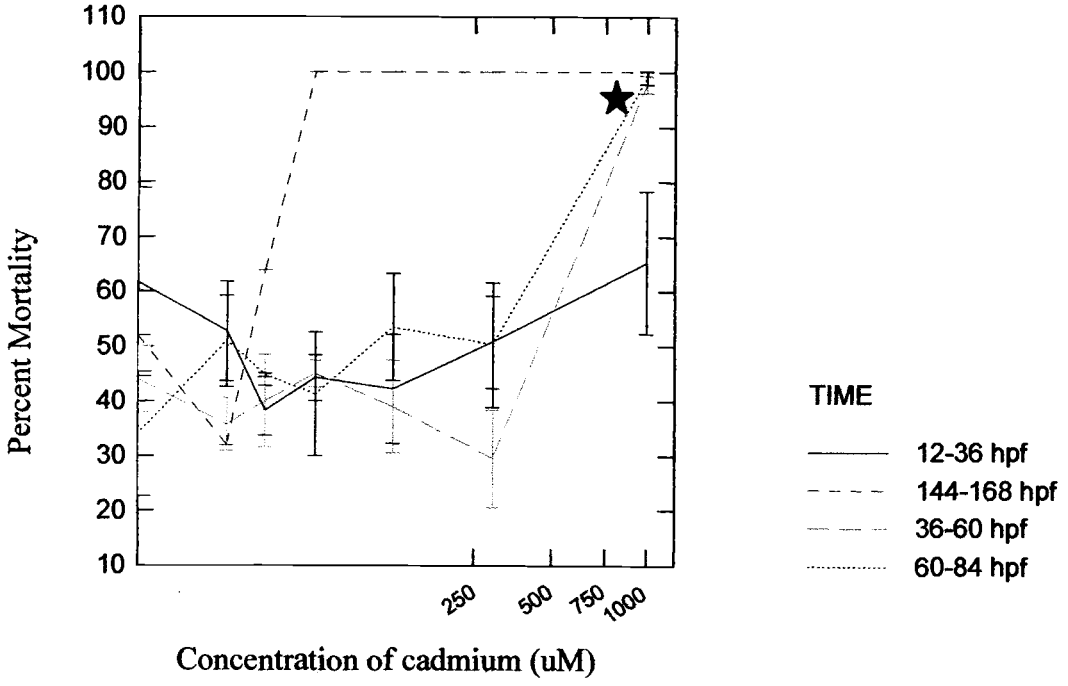

Table 3: Percent total mortality for Experiment I. The percentages for the fourth exposure period are of a single replication. Concentrations used are nominal.

	12-36hpf[B]	36-60hpf[C]	60-84hpf[D]	144-168hpf[E]
0µM	62%	44%	34%	52%
3µM	53%	36%	51%	38%
10µM	38%	40%	45%	64%
30µM	44%	45%	41%	100%
100µM	42%	39%	53%	100%
300µM	51%	29%	50%	100%
1000µM	65%	98%	99%	100%

Table 4. Results of a two-way ANOVA on skeletal deformities (α=0.05). There are three levels of time, and four levels of deformities (Caudal, Precaudal, Spinal Curvature, and Caudal/Precaudal).

	F-ratio	**P-value**
SD	49.899	<0.001
TIME	1.226	=0.295
TIME*SD	0.851	=0.532

Figure 3: Frequency of skeletal deformities in Experiment I. Stars denote P<0.01. Error bars use standard error.

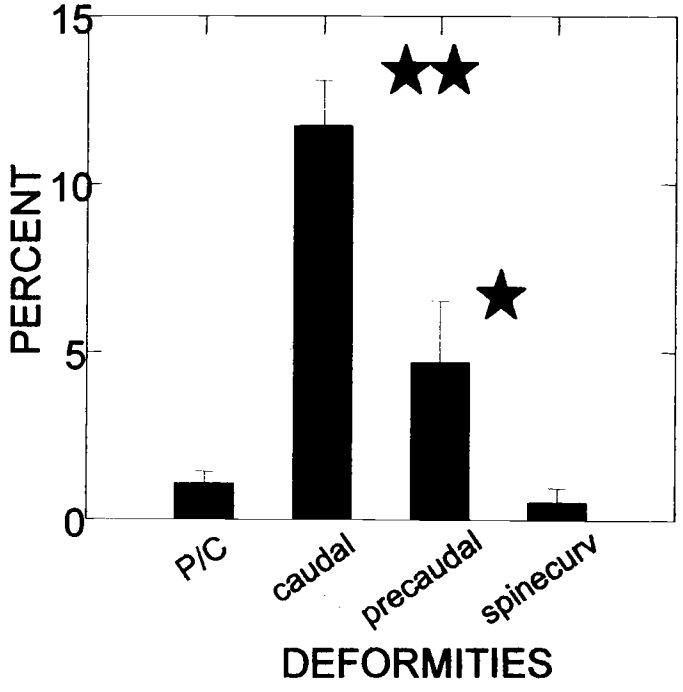

Table 5: Percentages of Skeletal deformities for Experiment I (n=919).

	Caudal	**Precaudal**	**Caud/precaud**	**Spinalcurv**	**Total**
N & H Spines	0.5%	1%	0%	0%	1.5%
Centra	11.5%	4%	1%	0.5%	17%
Total	12%	5%	1%	0.5%	18.5%

deformities was significantly different than the means for precaudal, caudal/precaudal, and spinal curvature ($P<0.01$ for each), likewise, the mean for precaudal deformities was significantly different that the means for caudal/precaudal, and spinal curvature ($P<0.01$ for each). The percentages of deformities were summarized in Table 5. There was no effect of Cd on incidence of any type of skeletal deformity at 40dpf ($P>0.05$ and $p>0.05$). LC50's for different exposure times and their upper and lower bounds were 809μM (923μM & 684μM) for 12-36hpf, 493μM (970μM & 304μM) for 36-60hpf, 398μM (891μM & 229μM) for 60-84hpf, and 11μM (15μM & 1μM) for 144-168hpf.

In Experiment II, pH was adjusted to pH 6 and dissolved Cd concentrations were measured by inductively coupled plasma spectrometry. Cadmium concentrations were consistently slightly higher than nominal (Table 6). Mortality at 10dpf was analyzed by one-way ANOVA (12-36hpf, and 60-84hpf, $P>0.05$)[E]. Data in Table 7 summarized percent mortality 10dpf . Two-way ANOVA examined 60dpf mortality Vs Cd concentrations ($F=7.606$, $P<0.001$)[E]. The mean mortality for 1000μM of Cd was significantly different than for other concentrations ($P<0.05$)[E]. There was no interaction between the times of exposure and Cd concentrations ($P<0.05$)[E]. One-way ANOVA determined no differences between exposure times for controls (caudal, $P>0.05$; precaudal, $P>0.05$)[E]; all controls were pooled to increase sample size. One-way ANOVA examined the effect of Cd concentration on caudal deformities (12-36hpf and 60-84hpf, $P>0.05$)[E]; likewise, there was no significant effect of Cd concentration on precaudal deformities (12-36hpf and 60-84hpf, $P>0.05$)[E]. Caudal and precaudal deformities were compared with one-way ANOVA ($P>0.05$)[E].

Table 6. Mean cadmium concentrations used in Experiment II ± standard errors (n=2).

Nominal (µM)	3	10	30	100	300	1000
Measured (µM)	3.7 ± 0.16	12.9 ± 0.40	42.4 ± 3.87	148.1 ± 28.02	405.2 ± 68.95	1152.0 ± 66.72
Nominal (mg/L)	0.337	1.12	3.37	11.2	33.7	112
Measured (mg/L)	0.418 ± 0.018	1.455 ± 0.045	4.765 ± 0.435	16.65 ± 3.15	45.55 ± 7.75	129.5 ± 7.5

Table 7: Mean percent mortality 10 days post fertilization in Experiment II (n=387; Appendix 2).[F]

	0µM	**3µM**	**10µM**	**30µM**	**100µM**	**300µM**	**1000µM**
12-36hpf	39%	40%	37%	33%	37%	40%	70%
36-60hpf	53%	37%	13%	47%	7%	20%	57%
60-84hpf	44%	42%	36%	31%	22%	40%	73%

Endnotes

A. 144-168hpf not analyzed because only one replication completed.
B. Chorion present on all embryos.
C. Chorion present on more that 90% of embryos.
D. Chorion present on more than 80% of embryos.
E. Chorion absent on all embryos.
F. 36-60hpf not analyzed because only two replications completed.

Chapter 4
Discussion

In preliminary studies Cd exposed larva with drastically bent tails were observed. This experiment was conducted for optimization of method for subsequent experiments. Abnormalities were characterized as bent tail, edema, and unhatched. Notochord lesions occurred after 12-36hpf exposures, and some embryo/larva from the 36-60hpf exposures exhibited dorsal or lateral flexure, or caudal truncation (very few). As the characterization bent tail did not distinguish between these abnormalities, no statistical comparisons were made. Chow and Cheng (2003) found that Cd exposure during gastrulation skewed developmental pathways of the notochord. This lead to abnormal morphological changes that resembled the notochord lesions found during somitogenisis in the Preliminary Experiment. Excessive handling involved in daily recording mortalities and abnormalities, and in photographing the abnormalities, contributed to extremely high mortality in this experiment. Consequently, it was deemed essential that daily manipulation was minimized for subsequent experiments aimed at fish survival until skeletons suitable for X-ray developed. Studies of notochord lesions were discontinued, and experimental methods were altered for improved survival.

Experiment I was conducted at circumneutral pH. The solubility of Cd is 14g/L at pH 7 (www.chemfinder.com). The exposure water was initially measured with a portable pH meter to be pH 7.1. Precipitation was apparent and probably included Cd complexed with minerals present in the embryo solution (0.14M NaCl, 5.4mM KCl, 0.25mM Na_2HPO_4, 0.44mM KH_2PO_4, 1.3mM $CaCl_2$, 1.0 mM $MgSO_4$, 4.2mM

$NaHCO_3$). Analysis by inductively coupled plasma spectrometry showed the precipitate to be Cd. A review of literature showed that 5mM of carbonate (CO_3) at pH 8.3 can reduce the solubility of Cd over a thousand fold (Bodek et al., 1988). In Experiment II, pH is adjusted to 6 and Cd remained in solution.

In Experiment I, two-way ANOVA showed a temporal difference in mortality (Table 2). At 1000μM Cd there was less mortality at the first exposure time than the other time periods. There was an apparent trend for higher mortality at lower Cd concentrations in the fourth exposure time (Table 3 and Figure 3). Since zebrafish hatched 2-3dpf, it was likely that the chorion protected the earlier stage embryo from toxicity. The protective role of the chorion for embryos, and the increased susceptibility of newly hatched larva were also found in newly hatched carp, *Cyprinus carpio* (Witeska et al., 1995). The 96 hour LC50's (page12) also showed a trend for higher Cd sensitivity at later exposure times. However, the wide range of the upper and lower bounds indicated higher sample sizes or lower variability data were necessary for increased precision of the LC50's.

While there was a difference in the type of deformities that occurred in Experiment I (n=919), it was not dependent on exposure time (Table 4). The descending order of deformities (Figure 4 and Table 5), caudal > precaudal > caudal/precaudal > spinal curvature, indicated low-level or no interference of early life stage Cd exposure in the formation of the axial skeleton. Neural and haemal spines of 40dpf zebrafish were not dense enough for effective X-ray imaging, so comparisons to deformed Willamette River fish (Markle et al., 2002) would be underestimated. Zebrafish were raised to 60dpf in Experiment II, but neural and haemal spines were

still indistinct when X-rayed. However, Hiraoka and Okuda (1984) reported that 14% centra deformities occurred at 0.01ppm Cd and 8% centra deformities occurred in the control; in Experiment I, centra deformities were 17% (12% caudal deformities and 5% precaudal deformities) for all Cd concentrations (Figure 4) and 20% (14.5% caudal deformities and 5.5% precaudal deformities) in the controls (not shown). Possibly early life stage skeletal development in medaka was more sensitive to Cd toxicity, or the retinal pigmentation stage in medaka perhaps occurred later in development of the axial skeleton than the corresponding stage in zebrafish.

Mild acidification of exposure solutions in Experiment II yielded dissolved Cd concentrations only slightly higher than nominal (Table 6). The lethality of Cd to embryonic zebrafish was strikingly lower at pH 6 than at pH 7 (Tables 1 and 7). This suggested particulate Cd was much more toxic than dissolved Cd. Since embryonic zebrafish rested on the bottom of exposure vessels they were certainly exposed to extremely high local concentrations of Cd-rich precipitate. The resistance of embryonic zebrafish to dissolved Cd was surprising. Cutaneous respiration provides sufficient surface area for gas exchange in embryonic zebrafish (Hughs et al., 1986; Dornesco and Coucou, 1974). The gill of fish is the target organ for most acute metal toxicity (Walker et al., 1977). Developing buds of the gill filament are present in 72hpf zebrafish (Kimmel et al., 1995). Absence of functional gill tissue in zebrafish embryos during early developmental periods perhaps explained the resistance to dissolved Cd.

In Experiment II, there was no effect of any Cd concentration at any exposure time on incidence of skeletal deformities (n=387). Unlike Experiment I, there was no

difference observed between the types of deformities. Also, data obtained were highly variable (not shown). There was a marked reduction in spawning observed in the broodstock (probably due to an infectious agent), and a corresponding increase in early embryo/larva mortality (not shown). Because of low egg availability, only two replications of 36-60hpf and no replications of 144-168hpf were obtained; consequently, the 36-60hpf exposures were not examined by ANOVA. Incidence of skeletal deformities in controls for different exposure times (12-36hpf, 36-60hpf, and 60-84hpf) were not different, so were pooled in all analyses of skeletal deformities.

In conclusion, typically lethal notochord lesions were observed in zebrafish embryos exposed to Cd at 12-36hpf. There was higher mortality from Cd toxicity at later exposure times. The chorion probably provided protection at earlier exposure times. At circumneutral pH, Cd solubility was reduced by mineral complexation, and Cd precipitated; particulate Cd (circumneutral pH) was more toxic to early life stages than dissolved Cd (pH 6). At circumneutral pH 1000μM Cd was significantly more toxic, and caused 100% mortality in embryo/larva exposed 36-60hpf and 60-84hpf; whereas, at pH 6 embryo/larva exposed at the same times have respectively 57% and 73% mortality. Absence of functional gill tissue in zebrafish embryos perhaps explained the resistance to toxicity of embryos exposed to dissolved Cd. The difference in the types of skeletal deformities occurring, caudal > precaudal > caudal/precaudal > spinal curvature, suggested low-level to no interference of early life stage Cd exposures in the formation of the zebrafish axial skeleton. Developmental stages later than those tested here are perhaps more sensitive to Cd-induced damage of the axial skeleton.

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Appendices

Appendix 1: Mortality data of zebrafish larva 7 days post exposure (dpe) from preliminary experiment of abnormalities. Embryo/larva are exposed to cadmium 12-36, 36-60, and 60-84 hours post fertalization (hpf).

0uM Cd (columns 2-4 list # of dead)

dpe	12-36hpf	36-60hpf	60-84hpf
1	4	4	4
2	1	1	1
3	1	1	1
4	2	2	2
5	0	0	0
6	0	0	0
7	0	0	0
	20.80%	20.80%	20.80%

10uM Cd (columns 2-4 list # of dead)

dpe	12-36hpf	36-60hpf	60-84hpf
1	4	3	29
2	1	0	0
3	4	5	1
4	2	0	0
5	1	0	0
6	1	3	1
7	0	0	0
	27.10%	22.90%	64.60%

30uM Cd (columns 2-4 list # of dead)

dpe	12-36hpf	36-60hpf	60-84hpf
1	4	4	16
2	1	0	2
3	1	3	2
4	2	1	0
5	0	1	1
6	0	3	0
7	0	1	1
	16.70%	27.10%	45.80%

100uM Cd (columns 2-4 list # of dead)

dpe	12-36hpf	36-60hpf	60-84hpf
1	4	6	38
2	0	2	3
3	0	15	3
4	0	0	0
5	2	1	1
6	0	0	1
7	0	2	0
	12.50%	54.20%	95.80%

300uM Cd (columns 2-4 list # of dead)

dpe	12-36hpf	36-60hpf	60-84hpf
1	10	4	24
2	0	0	10
3	0	4	0
4	1	0	2
5	1	1	1
6	0	0	1
7	0	1	0
	25.00%	20.80%	79.20%

1000uM Cd (columns 2-4 list # of dead)

dpe	12-36hpf	36-60hpf	60-84hpf
1	20	48	48
2	0	0	0
3	0	0	0
4	1	0	0
5	0	0	0
6	2	0	0
7	2	0	0
	52.10%	100%	100%

Appendix 2: Mortality data of zebrafish larva 10 days post fertilization (dpf) from experiment II. Embryo/larva are exposed to cadmium 12-36, 36-60, and 60-84 hours post fertalization (hpf).

12-36hpf		36-60hpf		60-84hpf	
Cd Conc. (uM)	Percent Mortality	Cd Conc. (uM)	Percent Mortality	Cd Conc. (uM)	Percent Mortality
0	33%	0	73%	0	47%
0	13%	0	33%	0	47%
0	53%	3	33%	0	40%
0	60%	3	40%	3	40%
0	33%	10	27%	3	47%
3	13%	10	0%	3	40%
3	67%	30	40%	10	47%
3	13%	30	53%	10	27%
3	80%	100	13%	10	33%
3	27%	100	0%	30	53%
10	47%	300	13%	30	27%
10	13%	300	27%	30	13%
10	27%	1000	87%	100	20%
10	60%	1000	27%	100	20%
10	40%			100	27%
30	73%			300	20%
30	0%			300	80%
30	13%			300	20%
30	47%			1000	20%
30	33%			1000	100%
100	20%			1000	100%
100	70%				
100	47%				
100	93%				
100	20%				
300	40%				
300	20%				
300	70%				
300	73%				
300	20%				
1000	80%				
1000	53%				
1000	53%				
1000	93%				
1000	100%				