**Using an Integrated Approach to Evaluate Apoptosis as a Biomarker Response in Estuarine Fishes**

By

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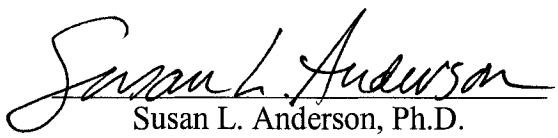

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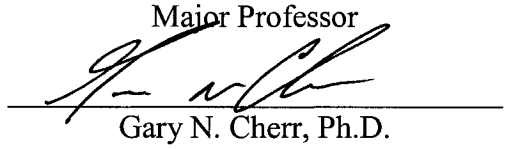

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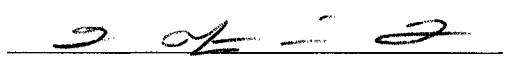

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## DEDICATION

To my parents, Cindy and Sheldon Rose, for their immeasurable love and support

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## ABSTRACT

Apoptosis, or programmed cell death, may be induced in fishes following exposure to a diversity of toxicants. While some apoptosis may prevent cancerous cell proliferation, the inappropriate stimulation of apoptosis by toxicants may lead to developmental and reproductive abnormalities. Little is known about the applicability of apoptosis as a biomarker in wild-caught fishes; however, the development of apoptosis as a biomarker is essential because it may be used as an early warning indicator for severe toxicant effects. Here, I used an integrated approach to evaluate apoptosis as a biomarker response in estuarine fishes. The longjaw mudsucker (*Gillichthys mirabilis*) was used in field investigations because it is a bottom-dwelling, salinity-tolerant goby found in tidal marshes. In the laboratory, topsmelt (*Atherinops affinis*) was used because it is an EPA-toxicity test organism that inhabits the same marshes as mudsuckers, and Cadmium (Cd) was chosen as a model contaminant because its toxicity has long been studied in fishes.

First, I determined whether TUNEL and caspase activity assays were reliable methods for measuring apoptosis as a biomarker response in estuarine fish from California tidal marshes varying in contamination levels. I measured increased apoptotic DNA fragmentation (TUNEL positive cells), and DEVDase activity (caspase-3 like protease activity) in hepatocytes of topsmelt exposed to non-cytotoxic Cd concentrations in the laboratory, indicating that TUNEL and caspase activity assays were sensitive apoptosis methods. In the field, apoptotic DNA fragmentation and DEVDase activity levels were significantly higher in the liver of longjaw mudsuckers from Stege Marsh (ST) relative to mudsuckers from the reference marsh. Moreover, average concentrations

of many sediment contaminants including metals, legacy organic chemicals, and pesticides were highest at ST, and apoptotic DNA fragmentation correlated with numerous sediment contaminants, suggesting that apoptosis was a reliable biomarker.

I then examined how increases in apoptosis and altered physiological responses were related to growth impairment of Cd-exposed larval topsmelt. Apoptotic DNA fragmentation and metallothionein-like protein levels were elevated, and Ca content of fish diminished at Cd concentrations that also impaired topsmelt growth. Oxygen consumption rates were correlated with growth impairment, and likely increased as a compensatory response to Cd exposure. These results indicate that less energy may have been allocated for growth because of an increased metabolic demand due to apoptosis, metallothionein synthesis, and changes in ion regulation. As part of this study, I applied otolith growth rate analysis to a more detailed investigation of Cd-impaired growth of topsmelt because growth measurements (i.e. final body size) used in standard toxicity tests with topsmelt were insensitive endpoints of toxicity. Otolith growth rate analysis was a useful method because it allowed for the detection of small differences in growth rates among treatments, even when differences in somatic growth were not observed.

This study demonstrates that apoptosis was a sensitive biomarker response because (i) liver apoptosis in wild-caught longjaw mudsuckers was correlated with environmental contamination, (ii) tissue- and concentration-dependent differences in apoptosis were found in Cd-exposed topsmelt, and (iii) apoptosis was associated with impaired growth of Cd-exposed fish. The use of apoptosis as an early warning indicator of ecologically relevant effects of toxicant exposure needs further exploration.

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# CHAPTER 1

Use of TUNEL and caspase activity assays to evaluate apoptosis as a biomarker response in estuarine fish

**Abstract**

I determined whether TUNEL and caspase activity assays were reliable methods for measuring apoptosis as a biomarker response in estuarine fish from California tidal marshes, varying in contamination levels. First, I assessed the sensitivity of TUNEL and caspase-3 activity assays in hepatocytes of cadmium (Cd)-exposed topsmelt (*Atherinops affinis*), a model marine species that inhabits the same tidal marshes as the bottom-dwelling longjaw mudsucker (*Gillichthys mirabilis*). Apoptotic DNA fragmentation levels, or TUNEL positive cells, and DEVDase activity, or caspase-3-like protease activity, were comparable to those of topsmelt hepatocytes exposed to cycloheximide (325 μM), a well-known apoptosis inducer. Increases in apoptotic DNA fragmentation levels were measured at low Cd concentrations (0.089 μM). At moderate Cd concentrations (0.89 μM), DEVDase activity and apoptotic DNA fragmentation levels were higher than those of control treatments, and necrosis was not observed, indicating that TUNEL and caspase activity assays were sensitive methods for measuring apoptosis. I then used TUNEL and caspase activity assays to assess differences in liver apoptosis of longjaw mudsuckers from diverse California tidal marshes and to compare apoptosis to other biomarker responses. Apoptotic DNA fragmentation and DEVDase activity were higher in fish from Stege Marsh (ST) relative to fish from Tom's Point (TP), the reference marsh. Average concentrations of many sediment contaminants including metals, legacy organic chemicals, and pesticides were highest at ST, and apoptotic DNA fragmentation was positively correlated with numerous sediment contaminants. Other biomarker responses including lipidosis, and glycogen depletion were elevated in the liver of fish from ST; however, neither lipidosis and glycogen depletion, nor DEVDase

activity correlated with sediment contaminant levels, indicating that these endpoints were less sensitive biomarkers than apoptotic DNA fragmentation. While my study demonstrates that apoptotic DNA fragmentation is a valuable biomarker response to contaminant exposure, future studies are needed to elucidate relationships between apoptosis and ecologically relevant effects of toxicant exposure.

**Introduction**

A diversity of biomarkers, or biological responses of organisms to toxicant exposure, is available for assessing the biological impact of environmental contamination (McCarthy and Shugart, 1990; Handy et al., 2003). Cytochrome P450 induction and activity have long been used to evaluate exposure of aquatic and terrestrial organisms to toxicants such as polycyclic aromatic hydrocarbons. Histopathological effects of toxicant exposure such as neoplastic lesions, glycogen deficiency, and macrophage aggregation within tissues also have been evaluated in toxicant-exposed organisms to determine whether structural changes within tissues were indicative of toxicant exposure (Teh et al., 1997). More recently, studies have begun to examine apoptosis, or programmed cell death, as an endpoint of toxicant exposure and effect in fishes and other vertebrates (Gavrieli et al., 1992; Sweet et al., 1999). Using histological evaluations and biochemical techniques, a wide range of toxicants were shown to induce apoptosis, indicating that apoptosis and biochemical changes associated with this process may be useful biomarker responses (Robertson and Orrenius, 2000). However, to my knowledge, apoptosis has not been rigorously applied to field investigations of toxicant-exposed organisms. While some apoptosis may prevent cancerous cell proliferation, the inappropriate stimulation of

apoptosis by toxicants may lead to developmental abnormalities, tissue dysfunction, or reproductive impairment (Raffray and Cohen, 1997). Thus, the development of apoptosis as a biomarker response is essential because it may be used as an early warning indicator for more severe toxicant effects such as impaired fitness. Moreover, the assessment of apoptosis together with multilevel biomarkers may provide the key to determining linkages between molecular biomarker responses and higher level effects of toxicant exposure.

Numerous methods have been established for measuring biochemical events associated with apoptosis including methods for measuring mitochondrial and cellular membrane changes, caspase cleavage and activation, apoptosis-related protein levels, apoptotic DNA fragmentation, and phosphatidylserine expression (Sweet et al., 1999). While these endpoints of apoptosis have been recommended as sensitive and informative biomarkers in fishes (Sweet et al., 1999), many apoptosis assays are only appropriate for studies of toxicant-induced apoptosis in cultured or isolated cells (e.g. phosphatidylserine expression), or studies using mammalian species (e.g. cell death receptor activation). A few of the apoptosis methods are suitable for field investigations because they may be used to assess apoptosis in most tissues or in tissue sections of fishes exposed to toxicants *in situ*. The terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay has been extensively used in the laboratory to measure endonuclease-dependent DNA fragmentation in tissues of fishes exposed to toxicants such as cadmium, PAHs, and dioxin (Gavrieli et al., 1992; Lundebye et al., 1999; Toomey et al., 2001; Weber and Janz, 2001). DNA gel electrophoretic techniques (Piechotta et al., 1999) and the diffusion assay (Frenzilla et al., 2004) also were used to examine apoptotic DNA

fragmentation in fishes exposed to toxicants in the laboratory. Assays that measure caspase-3 activation and activity have potential for field application because these methods have been applied to studies of apoptosis in tissues of fishes or mammalian species (Bajt et al., 2000; Lentz et al., 2003). While the assays described above were useful in laboratory investigations, little is known about their applicability and sensitivity in field investigations of toxicant-exposed fishes.

The overarching goal of this study was to evaluate the sensitivity and applicability of TUNEL and caspase activity assays for measuring apoptosis as a biomarker response in estuarine fish from contaminated California tidal marshes. I chose to assess caspase-3-like protease activity, or DEVDase activity, because it is an early event in the process of apoptosis that often plays a central role in toxicant-induced apoptosis (Sweet et al., 1999; Robertson and Orrenius, 2000). The TUNEL assay was of interest because it measures endonuclease-dependent DNA fragmentation, a late event in the apoptotic process, and numerous laboratory investigations have used the TUNEL assay to demonstrate toxicant-induced apoptosis in the liver of fishes (Sweet et al., 1999). First, I examined the sensitivity of TUNEL and caspase activity assays in hepatocytes of topsmelt (*Atherinops affinis*) exposed to cadmium (Cd), an environmentally relevant contaminant in California tidal marshes (Peter Green, personal communication), known to induce apoptosis in fishes (Piechotta et al., 1999; Risso-de Faverney et al., 2004). Topsmelt were used because they are well-known as sensitive species in EPA-toxicity tests, and they inhabit the same California tidal marshes as longjaw mudsuckers (*Gillichthys mirabilis*), a bottom-dwelling fish used in my field investigations. I used topsmelt in place of longjaw

mudsuckers in the controlled study such that small local mudsucker populations would not be reduced because of these experiments.

My second objective was to determine if TUNEL and caspase activity assays were reliable methods for detecting differences in liver apoptosis of longjaw mudsuckers among California tidal marshes with diverse biogeographical characteristics and varying levels of contamination. As part of this objective, I compared apoptosis to other biomarker responses (i.e. cytochrome P4501A, histopathological effects) measured in livers of the same fish. Here, I used the longjaw mudsucker as a model estuarine species because it is a sulfide and salinity tolerant goby that: (1) is found in tidal marshes from Southern to Northern California, (2) spends most of its time in contact with tidal marsh sediments, and (3) has a small home range within a marsh (de Vlaming, 1972). I focused on apoptosis in liver because it is the major organ of biotransformation for many toxicants, in which toxicant-induced apoptosis has been shown to occur (Hinton, 1994; Robertson and Orrenius, 2000).

## Methods

### *Apoptosis in Cd-Exposed Topsmelt Hepatocytes*

*Topsmelt Hepatocyte Isolation and Culture.* Juvenile topsmelt (4-5 g) from Aquatic Bio Systems (Fort Collins, CO) were maintained in a flow-through seawater system at 12-14°C. Fish were fed Tetramin Flakes daily and acclimated for at least two weeks. Hepatocyte isolation was performed according to van den Hurk (1998) with a few modifications. For each treatment, livers from three fish were dissected and hepatocytes isolated by trypsinization and mechanical separation over a 100 μM mesh. Cells were

seeded at 5 x $10^6$ to 1 x $10^6$ cells/mL in RPMI media supplemented with 10% FBS, 2 mM L-glutamine, 50 units/ml Penicillin, 50 μg/mL Streptomycin, and 14 mM HEPES buffer. Cells were maintained in 24-well plates and incubated under atmospheric air at 20°C for 2 h before experimentation. Cell number and viability were assessed using the trypan blue exclusion assay according to standard protocols (Brus and Glass, 1973).

*Exposure of Topsmelt Hepatocytes*. Cd stock solutions were prepared by adding $CdCl_2$ to 0.9% NaCl. Each test solution was prepared by adding a Cd stock solution to RPMI media supplemented with 1% essential amino acids instead of serum. Cycloheximide (CHX, BioVision, Inc., Mountain View, CA), a well-known apoptosis inducer, also was used in this study for comparative purposes. Test solutions of CHX were prepared by diluting stock solutions, prepared in DMSO, into RPMI media supplemented with 1% essential amino acids. For necrosis/cytotoxicity assays, primary liver cell cultures of topsmelt hepatocytes were exposed to Cd (0.05 to 50 μM) or to 0.9% NaCl (control) for 12 and 24 h. For determination of apoptosis using the TUNEL assay, cells were exposed to 0.9% NaCl, Cd (0.089 to 8.9 μM), and CHX (325 μM) for 12 or 24 h. For determination of apoptosis using caspase activity assays, topsmelt hepatocyte cultures were exposed to Cd (0 to 8.9 μM) or CHX (325 μM) for 1, 4, 8, 12, and 24 h. To evaluate whether Cd-induced apoptotic DNA fragmentation (TUNEL positive cells) was dependent on DEVDase activity, TUNEL and caspase activity assays were performed on hepatocytes exposed to Cd (8.9 μM) or CHX (325 μM) for 12 h, in the presence and absence of the Z-VAD-FMK caspase family inhibitor (2 μM, BioVision, Inc., Mountain View, CA).

*Necrosis of Cd-Exposed Hepatocytes*. I used the trypan blue exclusion assay and Sytox green uptake (Molecular Probes, Inc., Eugene, OR) to evaluate necrosis, or cells with reduced membrane integrity, of topsmelt hepatocytes exposed to Cd for 12 and 24 h. For Sytox green uptake, cells from each treatment were transferred to a 96-well plate. Sytox green (500 nM) was added to each treatment and cells were incubated for 20 min. at room temperature (RT). Fluorescence was examined at 490 nm (ex) and 530 nm (em) using a SpectroMax fluorescence microplate reader (Molecular Devices Corporation, Sunnyvale, CA).

*Apoptosis of Cd-Exposed Hepatocytes*. The TUNEL assay was used to examine apoptotic DNA fragmentation in topsmelt hepatocytes exposed to Cd or CHX using modifications of Gavrieli et al. (1992) and a commercial kit, the Dead-End Fluorometric TUNEL System (Promega, Madison, WI). Briefly, hepatocytes were transferred to poly-l-lysine coated slides, and fixed in 10% methanol-free formalin for 25 min. at 4°C. Slides were rinsed in PBS and permeabilized in 0.2% Triton X-100. Slides were incubated with terminal deoxynucleotidyl transferase (TdT) equilibration buffer for 10 min. at RT. The equilibration buffer was replaced with 50 μL of TdT reaction mixture, which contained TdT enzyme, equilibration buffer, and a nucleotide mix with fluorescein-12-dUTP, and incubated at 37°C for 1 h. The reaction was stopped by immersing the slides in 2X SSC for 15 min., and the slides were rinsed three times in fresh PBS containing 0.1% Triton ® X-100 and 5mg/mL of BSA, and one time in PBS alone. After rinsing in double deionized water, slides were air dried and mounted in a medium containing 90% glycerol, 10% PBS, 0.2% n-propyl gallate. Slides were stored at -20°C for 1-2 days until further analysis. An Olympus BX50WI microscope (Olympus America, Melville, NY) with a

standard fluorescein filter set was used to view TUNEL positive nuclei fluorescence. Fluorescent images were compared to light images of each field to determine the percent of cells that were TUNEL positive. TUNEL positive cells had nuclei with at least 125% of the background fluorescence of control cells. At least 50 cells were examined per replicate for DNA fragmentation. Two-way ANOVA and Student-Newman-Keuls (SNK) multiple comparisons were used to examine differences in TUNEL positive cells among treatments and between time periods (Sokal and Rohlf, 1981).

Caspase-3-like protease activity, or DEVDase activity, was evaluated in topsmelt hepatocytes exposed to Cd or CHX using the Apo-ONE™ Homogenous Caspase-3/7 Assay (Promega, Madison, WI). Cells (50 μL) were transferred to 96-well plates and 50 μL of homogeneous caspase 3/7 reagent (substrate and buffer mixture) was added to all wells. Plates were mixed and incubated for 60 min. at 30 °C. Measurements were taken on a SpectroMax fluorescence microplate reader at 485 nm (ex) and 530 nm (em), every 3 min. for at least 30 min. Two-way ANOVA and SNK multiple comparisons were used to detect differences in DEVDase activity among treatments and among time periods.

*Apoptosis in Liver of Field-Collected Longjaw Mudsuckers*

*Sampling Strategy.* This study was part of a large, multi-disciplinary program, the Pacific Estuarine Ecosystem Indicator Research consortium (PEEIR), designed to develop indicators of wetland ecosystem health through assessments of stressor-response patterns in wetland biota. In this investigation, I evaluated apoptosis in liver of longjaw mudsuckers in relationship to pollutant levels in five California tidal marshes: Walker Creek (WC) and Tom's Point (TP) in Tomales Bay, Stege Marsh (ST) and China Camp

(CC) in San Francisco Bay, and Carpinteria Salt Marsh (CS) in Southern California (Fig. 1). Tidal marshes were chosen in part because longjaw mudsuckers were abundant, and contaminant levels varied among and within marshes. In addition, as part of PEEIR these marshes were selected because they span large biogeographic ranges and vary morphologically, which enabled me to test the reliability of apoptosis as a biomarker, across dissimilar environments.

*Fish and Tissue Handling*. In the August of 2003, up to 12 longjaw mudsuckers were sampled from multiple stations within each of the five tidal marshes using minnow traps and beach seines. Fish were transported live to either UC Santa Barbara (S. California sites) or to the Bodega Marine Laboratory (N. California sites) and sacrificed by severing the spinal cord. Weight, length, and liver weight were recorded and liver was removed and sectioned into three pieces when possible. One portion of liver was randomly placed into 10% methanol-free formalin for the TUNEL assay, and for histopathological analyses. In addition, depending on the tissue size, one to two liver portions were randomly placed in microfuge tubes and quick frozen in liquid nitrogen for cytochrome P4501A protein analysis and caspase activity assays.

*Apoptosis Assays*. For the TUNEL assay, liver was fixed in 10% methanol-free formalin for 48 hrs. Samples were rinsed two times in PBS for 10 min. and held in PBS at 4°C for 1 hr. Samples were transferred to 50% ethanol, held at 4°C for 1 hr, and transferred to 75% ethanol. Samples were further dehydrated, embedded in paraffin, and sectioned according to standard protocols by Central Histology Services (Sacramento, CA). Slides of liver sections were examined for apoptotic DNA fragmentation using the TUNEL assay with a few differences in the method described above. Briefly, deparaffinized and

rehydrated liver sections were incubated with 20 ng/mL proteinase K for 30 min. at 37°C, and rinsed with PBS. Fish sections were incubated with the TdT reaction mixture, and the reaction was stopped as described above. The slides were then counterstained with Hoechst 33258 solution (Molecular Probes, Inc., Eugene, OR) freshly diluted to 0.1μg/mL in PBS for 15 min. at RT in the dark. After rinsing in double deionized water, slides were air dried, mounted in media, and stored at -20°C for 1-2 days until further analysis. An Olympus BX50WI microscope (Olympus America, Melville, NY) with a standard fluorescein filter set was used to view the green fluorescence of TUNEL positive nuclei and the blue fluorescence of the Hoechst stain, which stains all cell nuclei, was detected using a DAPI/Hoechst filter at approximately 460 nm. Images of all nuclei (blue fluorescence) were overlaid with images of TUNEL positive cells in each field to determine the percent of cell nuclei that were TUNEL positive. At least 300 liver cells, excluding red blood cells, were analyzed from at least five images from each fish liver to determine the percent of TUNEL positive cells.

Caspase-3-like activity or DEVDase activity, was assessed in livers of mudsuckers according to a modified method of Bajt et al., (2000). Liver samples (200 mg) were thawed on ice and 400 μL of hypotonic lysis buffer was added to each sample. Liver samples were homogenized for 25 sec. on ice, sonicated for 25 sec. on ice, and centrifuged for 20 min. at 14,000 x *g* at 4°C. The supernatant was removed and placed on ice. A subsample of each supernatant was used to determine protein concentrations according to the BCA protein assay (Pierce, Rockford, IL). Another subsample with approximately 100 μg of protein was used to assess DEVDase activity using the commercially available kit, the Fluorometric CaspACE™ Assay System (Promega,

Madison, WI) with Z-DEVD-AMC as the fluorescent substrate. Measurements of AMC release from the peptide were taken on a SpectroMax fluorescence microplate reader at 360/460 nm (ex/em) every 5 min. for at least 45 min. The fluorescence detected was converted to pmol based on a AMC standard curve. Average change in fluorescence was calculated, and the average DEVDase activity in pmol per min. was normalized to the amount of protein in each well (~100 μg).

*Additional Liver Biomarkers*

Cytochrome P4501A (CYP1A) protein levels were examined in frozen liver sections of longjaw mudsuckers. Briefly, frozen livers (~25 mg) were homogenized on ice and proteins electrophoresed on a 10% SDS-PAGE gels. CYP1A proteins were detected using western blotting with a rabbit anti-P4501A IgG primary antibody, a goat anti-rabbit HRP secondary antibody, and ECL™ western blotting detection reagents (Amersham BioSciences, UK). CYP1A protein levels were visualized on an Epi-chemi dark room instrument (UVP Laboratory Products, Upland, CA) quantified using Labworks™ Analysis Software as the optical density of each sample band (relative to the control band) normalized to liver wet wt (mg) of the sample. CYP1A protein analysis will be reported in more detail (by station within in marsh) in a companion paper.

For histopathological analyses, livers were sectioned as described above and stained with hematoxylin and eosin (H&E). Histopathological evaluations were performed on blind coded liver sections using semi-quantitative histopathological techniques (Bennett et al., 1995). Liver was examined for the presence and severity of each of the following effects: glycogen depletion, lipidosis, macrophage aggregates,

single-cell necrosis, pericholangial lymphocytes, foci of cellular alteration, Ito or stellate cell proliferation, and vacuolated spaces. Lesions were scored as none (0), mild (1), moderate (2), and severe (3).

*Sediment Chemistry*

In July and August of 2003, 80 sediment samples were collected from stations within five tidal marshes (CC, CS, ST, TP, WC). Composite sediment samples were taken from two to three elevations (creek channel, creek bank, and marsh edge) at each station within a tidal marsh, depending on marsh geology. The composite sediment sample was comprised of three samples taken within a 1 m area around each station using a Nalgene plastic corer to remove the top 15 cm of sediment. The sediment composite sample was immediately placed in a glass jar on ice, and stored at –20°C. Thawed and homogenized subsamples (20 g) were analyzed for metals and pesticides and the remaining sample in the glass jar was analyzed for legacy organic chemicals.

For analysis of 27 elements, sediment samples (50 mg) were digested by a hot, open digestion with nitric acid and perchloric acid according to standard methods (Fan et al., 2001). The final digest (approximately 100 μL) was removed of insoluble residues and diluted with 0.1 N nitric acid to a final volume of 10 mL. Samples were brought to room temperature, gently stirred to return condensed vapor to the solution in the bottom of the tube. Samples (250 μL) were diluted 20-fold in 1% nitric acid (Trace Metal grade, Fisher Scientific, Pittsburgh, PA) in a new polypropylene centrifuge tube. A blank tube was measured as a control, confirming negligible contamination. Quantification was by direct aspiration in an ICP-MS (model 7500i, Agilent, Palo Alto, CA) and compared to

external standards (traceable to NIST) covering a range from 0.1 μg/L to 20 μg/L. In the fashion prescribed by EPA method 6020, the instrument detection limit (triple the standard deviation of blanks) was 0.006 μg/L corresponding to a method detection limit of 0.12 μg/L. For each of the 27 elements analyzed, all major isotopes were quantified and all possible interfering elements were determined to ensure negligible interference. No interference corrections were necessary. The instrument was operated with a robust plasma characterized by 0.4% CeO/Ce.

For analysis of legacy organic chemicals, sediments were extracted with dichloromethane using Soxhlet extraction techniques. Extracts were cleaned using alumina column chromatography to remove interfering compounds, and legacy organic chemicals including PAHs, PCBs, DDT, DDD, DDE, nonachlor, and chlordanes were analyzed by GC/MS according to (Hwang et al., 2003).

Sediment samples were analyzed for current-use pesticides by the U.S. Geological Survey organic chemistry laboratory (Sacramento, CA). Samples were solvent-extracted and analyzed using a Saturn 2000 GC/MS ion trap system (Varian, Inc., Walnut Creek, CA), for 32 current-use pesticides.

*Statistical Analyses*

Data was transformed when necessary and one-way fixed factor ANOVA and SNK's multiple comparisons were used to examine differences in TUNEL positive liver cells and DEVDase activity, separately, among marsh stations (Sokal and Rohlf, 1981). One-way ANOVA and SNK's multiple comparisons also were used to examine differences in CYP1A protein levels, and histopathological endpoints (e.g. glycogen

depletion, lipidosis) in the liver of longjaw mudsuckers among tidal marshes, for each endpoint separately. Data was transformed and Pearson Product Moment Correlations were used to evaluate the relationship between TUNEL positive liver cells and liver DEVDase activity in longjaw mudsuckers from all tidal marshes sampled. Correlations also were performed to evaluate the strength of the relationship between the percent of TUNEL positive cells in mudsucker livers vs. levels of metals, legacy organic contaminants, and pesticides in sediment from stations within marshes in this study. Similar correlations were performed to examine relationships between DEVDase activity, CYP1A protein levels, lipidosis, and glycogen depletion, separately, vs. levels of sediment contaminants.

## Results

*Apoptosis in Cd-Exposed Topsmelt Hepatocytes*

Necrosis or cytotoxicity increased with increasing Cd concentration as measured by the trypan blue exclusion assay and Sytox green nuclear stain fluorescence. The mid-point cytotoxicity ($EC_{50}$) values at 12 and 24 h, determined from probit-transformed mortality data, were 15.2 μM and 5.3 μM Cd, respectively. A highly significant correlation was detected between the two cytotoxicity endpoints (Pearson Product Moment Correlation, $R=0.99$, $P<0.0001$).

The intensity of fluorescence in TUNEL positive cells and the percent of TUNEL positive cells increased with increasing Cd concentration and from 12 to 24 h (Two-way ANOVA, $P<0.05$, Fig. 2). At 12 h post-exposure, levels of TUNEL positive cells ranged from $14.4 \pm 6.8$ % ($\pm$ 1 SE) in the control group to $80.5 \pm 2.6$% in the 8.9 μM Cd

treatment ($P<0.05$), which was comparable to that of the CHX-treated hepatocytes (77.2 $\pm$ 7.1%). By 24 h, significant levels of TUNEL positive cells were detected in the 0.089 μM Cd treatment (43.5 $\pm$ 7.9%) relative to the controls and both were lower than those of the highest Cd and CHX treatments ($P<0.05$).

DEVDase activity of Cd-exposed hepatocytes was dependent on concentration and time (Two-way ANOVA, $P<0.001$, Fig. 3). DEVDase activity in hepatocytes exposed to 8.9 μM Cd was higher than that of controls from 4 to 12 h post-exposure ($P<0.05$). Hepatocytes exposed to 0.89 μM Cd demonstrated lower DEVDase activity than controls at 1 h post-exposure and were significantly higher than controls by 12 h ($P<0.05$). At 24 h post-exposure, DEVDase activity in hepatocytes exposed to 8.9 μM Cd had diminished to control levels while DEVDase activity in hepatocytes exposed to 0.89 μM remained higher than controls at $P=0.10$. DEVDase activity levels in hepatocytes exposed to 0.089 μM Cd were comparable to controls from 1 to 24 h post-exposure while DEVDase activity in CHX-exposed hepatocytes was greater than controls from 8 to 24 h ($P<0.05$).

I also examined whether the inhibition of caspases would lead to diminished levels of apoptotic DNA fragmentation, or TUNEL positive cells. The presence of the caspase inhibitor led to a reduction in the percent of TUNEL positive cells in the CHX treatments ($P<0.05$), but not in the Cd treatment, relative to the treatments without the inhibitor (Fig. 4).

*Apoptosis in Liver of Field-Collected Longjaw Mudsuckers*

Average concentrations of various metals (Ag, As, Cd, Cu, Pb, Se, Zn), organic chemicals (PAHs, PCBs, p,p'-DDD), and pesticides (Eptam, Pebulate, Molinate, Cycloate, Promethryn, Methidathion, Napropamide) in sediment from ST were higher than contaminant concentrations in sediment from other marshes (Table 1). Many contaminants in sediment from ST were at least 4-fold higher than those in sediments from the reference site, TP, including Ag, Cu, Pb, Zn, total PAHs, total PCBs, p,p'-DDD, Eptam, Pebulate, Molinate, Cycloate, Promethryn, Methidathion, and Napropamide.

The percent of TUNEL positive cells in the liver of fish from all stations at ST, WC, and CC were higher than those of fish from TP; however, only fish from stations M, NOP, and RS at ST had significantly higher levels of TUNEL positive cells than those of fish from TP stations at $P<0.05$ (Fig. 5). No significant differences in DEVDase activity were found among stations within marshes. Regardless of station, the percent of TUNEL positive cells and lipidosis in liver of longjaw mudsuckers from ST were significantly higher than those of fish from TP, the reference marsh (ANOVAs, $P<0.01$, Figs. 6,7). DEVDase activity in liver of fish from ST was significantly higher than DEVDase activity in fish from TP at $P=0.06$ (ANOVA, Fig. 7), and glycogen depletion in liver of fish from ST was higher than in fish from TP at $P=0.11$. Levels of CYP1A proteins, and some histopathological effects in the liver (macrophage aggregates, single-cell necrosis, pericholangial lymphocytes, foci of cellular alteration, Ito or stellate cell proliferation, and vacuolated spaces) of fish from ST were not significantly higher than those of fish from TP.

The proportion of TUNEL positive liver cells of longjaw mudsuckers were somewhat correlated with liver DEVDase activity levels (Fig. 8), but were not correlated with other biological responses in liver of mudsuckers from stations of all marshes. Levels of TUNEL positive liver cells of longjaw mudsuckers also were positively correlated with levels of four metals (Al, Cu, As, and Se) and to two legacy organic chemicals, p,p'-DDD and total PCBs, in sediment from stations of all marshes (Fig. 9). DEVDase activity in liver of mudsucker did not correlate with sediment contaminant levels or with other biological responses assessed.

## Discussion

*Apoptosis in Cd-Exposed Topsmelt Hepatocytes*

The results of this study demonstrate that Cd exposure led to apoptotic DNA fragmentation, measured as TUNEL positive cells, of topsmelt hepatocytes that was dependent on concentration and time. The 12 h exposure to 0.89 μM Cd, a concentration substantially lower than the 12 and 24 h midpoint cytotoxicity levels, led to increased levels of DEVDase activity and apoptotic DNA fragmentation that were comparable to those of CHX-exposed hepatocytes. Previous studies using both fish and mammalian models also have shown that Cd exposure led to increased DEVDase activity and apoptotic DNA fragmentation, or endpoints indicative of apoptosis (Robertson and Orrenius, 2000). Increased levels of DEVDase activity and apoptotic DNA fragmentation, were measured in rainbow trout hepatocytes exposed to Cd (1-10 μM) for 6 to 48 h (Risso-de Faverney et al., 2001a). Similarly, Cd (10 μM ) exposure led to cellular changes characteristic of apoptosis in Rat-1 fibroblasts including DNA

fragmentation, phosphatidylserine externalization, and caspase activation (Kim et al., 2000).

At the highest concentration (8.9 μM), Cd likely caused both apoptosis and necrosis of topsmelt hepatocytes. While hepatocytes exposed to 8.9 μM Cd for only 12 h demonstrated higher levels of DEVDase activity and apoptotic DNA fragmentation than those of controls, necrosis of hepatocytes was not significant, suggesting that most hepatocytes were apoptotic. In contrast, hepatocytes exposed to 8.9 μM Cd for 24 h demonstrated levels of necrosis that were at least 3-fold higher than those of controls and while levels of apoptotic DNA fragmentation were higher than controls at this Cd concentration, DEVDase activity had diminished to control levels. Because the TUNEL assay does not always differentiate between necrotic and apoptotic cell death (Grasl-Kraupp et al., 1995), it is possible that the DNA fragmentation I detected was occurring in both necrotic and apoptotic cells and that after 24 h most cells were necrotic. Furthermore, Cd is known to cause both apoptosis and necrosis. Under sulfhydryl deficiency, 0.3 μM Cd induced apoptosis while higher Cd concentrations led to necrosis in the H4IIE rat-derived hepatocyte cell line (Kim et al., 2003).

I found that the presence of the caspase inhibitor led to a reduction in apoptotic DNA fragmentation levels in the CHX treatments, but not in the Cd treatment, indicating that Cd-induced apoptosis may be induced through a caspase-independent pathway. In Hep3B cells, Cd-induced apoptosis was not dependent on caspase activity, but likely was the result of calcium- and oxidative stress-related impairment of mitochondria, and the release of the apoptosis-inducing factor (Lemarié et al., 2004). Apoptotic DNA fragmentation levels also may have been unaffected by the inhibition of DEVDase

activity because TUNEL was detecting DNA fragmentation in necrotic as well as apoptotic cells (Grasl-Kraupp et al., 1995). Cd (100 μM) exposure led to caspase-independent apoptosis and necrosis of normal human lung cells, MRC-5 (Shih et al., 2003).

The TUNEL and caspase activity assays were sensitive methods for detecting Cd toxicity in topsmelt hepatocytes, indicating that these assays were applicable as biomarker techniques for examining toxicant-induced apoptosis in estuarine fishes. DNA fragmentation, as measured by the TUNEL assay, was a sensitive endpoint of Cd toxicity; increases in apoptotic DNA fragmentation levels occurred at concentrations as low as 0.089 μM Cd. DEVDase activity corresponded well with apoptotic DNA fragmentation, but not with necrosis or loss of membrane integrity, suggesting that it was a specific indicator of apoptosis. On the other hand, it is notable that there was high variability in DEVDase activity, even though this was a highly controlled laboratory study. This suggests that DEVDase activity may be a less reliable biomarker response than apoptotic DNA fragmentation.

*Apoptosis in Liver of Field-Collected Longjaw Mudsuckers*

To my knowledge, this is the first study to successfully apply apoptosis as a biomarker response in fish from a broad range of field sites, varying in environmental contamination, biogeography, and morphology. By using a rigorous approach, I demonstrated that the TUNEL assay was a sensitive method for detecting liver apoptosis in wild-caught fish in response to environmental contamination, and caspase activity assays were useful for supporting the findings of the TUNEL assay. Specifically, I

measured higher levels of liver apoptosis in fish from a contaminated marsh (ST) relative to those of fish from the reference marsh (TP), despite variability due to environmental stressors such as salinity, temperature, oxygen concentrations, nutrient loading, and levels of suspended sediment. Moreover, my preliminary data indicates that the reliability of the TUNEL assay is unaffected by temporal variability; higher levels of apoptotic DNA fragmentation were found in fish from ST relative to fish from TP in the summer of 2002 (data not shown) as well as in the summer of 2003. Recent field studies have assessed apoptosis in fish, along with other biomarker responses; however, none to date have provided strong evidence that levels of apoptosis were higher in fish from contaminated sites relative to those from reference sites. Using the diffusion assay, variation in erythrocyte apoptosis in eelpout (*Zoarces viviparus*) was more strongly related to seasonal variation, than oil contamination levels (Frenzilla et al., 2004). Differences in ovarian apoptosis were not detected using 3'end-labeling in black bullhead *(Ameiurus melas*) and bluegill sunfish *(Lepomis macrochirus*) from metal-contaminated sites relative to those from reference sites (Yoo and Janz, 2003). This study differs from previous field investigations because we: (1) investigated apoptosis in the liver, because it is the major organ of biotransformation and metabolism, susceptible to toxic metabolic products, and a tissue in which toxicant-induced apoptosis occurs (Hinton, 1994; Piechotta et al., 1999); (2) used TUNEL and caspase activity assays to assess apoptosis; and (3) provided evidence that liver apoptosis was higher in fish from contaminated tidal marshes, despite variability due to non-contaminant related factors.

I demonstrated that apoptotic DNA fragmentation, measured as TUNEL positive cells, was a highly sensitive and informative biomarker of contaminant exposure and

effect in fish. The highest levels of apoptotic DNA fragmentation were found in the liver of mudsuckers from ST, in which average levels of numerous metals, organic chemicals, and pesticides in sediment were higher than those from other marshes, including TP, the reference marsh. It is probable that the longjaw mudsuckers evaluated in this study were directly exposed to these sediment contaminants, because mudsuckers are bottom-dwelling fish, that scavenge for food and burrow in the mud (de Vlaming, 1972). I also determined that apoptotic DNA fragmentation levels were positively correlated with six of the sediment contaminants analyzed. These correlations indicate that apoptotic DNA fragmentation levels in longjaw mudsucker livers reflect varying contamination levels at stations within all of the tidal marshes examined, including TP, the reference marsh, moderately contaminated marshes such as CC and CS, and ST, the highly contaminated marsh.

The high, but non-significant levels of apoptotic DNA fragmentation in fishes from CC relative to those of fishes from the reference marsh may be explained in part by the unique biogeographical features of CC; fewer mudsuckers inhabited this marsh relative to other marshes, and thus I had a much lower sample size at CC (n=5) as compared to other marshes in this study (n>18 per marsh). In addition, one of the five fish at CC had unexplainably high levels of TUNEL positive liver cells (~7%). Because the liver typically does not have high background levels of apoptosis (Gavrieli et al., 1992), it is likely that this fish was dying at the time of sampling, or that the TUNEL positive cells measured in this fish were false positives (Grasl-Kraupp et al., 1995).

In this study, DEVDase activity was marginally higher in liver of mudsuckers from ST relative to that of fish from TP, and DEVDase activity was weakly correlated

with apoptotic DNA fragmentation levels. However, in contrast to apoptotic DNA fragmentation, DEVDase activity did not correlate with sediment contaminant levels. These results indicate that caspase activity assays may be most useful when applied in conjunction with other apoptosis biomarkers to support the findings of apoptosis biomarker assays and to verify that apoptosis was occurring in response to toxicant exposure. These findings also indicate that caspase-3-like activity was a highly variable response with lower sensitivity than apoptotic DNA fragmentation. The high variability in caspase-3-like activity may be due to a low signal-to-noise ratio in DEVDase activity; caspase-3 substrates may be cleaved by other caspase isoforms and possibly by non-specific proteases (Yu et al., 2001). Thus, differences in DEVDase in fishes among tidal marshes in the present study may have been obscured by the high variability associated with the caspase-3 activity method.

Numerous contaminants found at ST may have led to an increase in apoptotic DNA fragmentation in the liver of longjaw mudsuckers. Elevated levels of metals in sediment from ST, including Cd and copper (Cu) may have contributed to increased levels of apoptosis in ST fish. Environmentally relevant concentrations of Cu induced apoptosis in the olfactory epithelium of rainbow trout (Julliard et al., 1996), and Cd induced apoptosis in liver cells of fishes (Piechotta et al., 1999; Risso-de Faverney et al., 2004). Legacy organic chemicals including PCBs and PAHs in ST sediment also may have contributed to elevated apoptosis in mudsucker livers. PCBs were shown to induce apoptosis in the liver of dab (Piechotta et al., 1999) and likely contributed to increased DEVDase activity in cultured trout hepatocytes (Risso-de Faverney et al., 2001b). Exposure to PAHs led to apoptosis of skin and gonadal cells of pink salmon larvae

(*Oncorhynchus gorbuscha*) (Marty et al., 1997), and increased apoptosis in juvenile channel catfish (*Ictalurus punctatus*) ovaries (Weber and Janz, 2001). Exposure of fish to current-use pesticides at ST may have led to increased apoptosis. However, little is known about pesticide-induced apoptosis in fishes or mammalian species.

Non-apoptosis biomarker responses also were elevated in the liver of longjaw mudsuckers from ST, relative to those of fish from TP, providing additional evidence for functional damages in fish from ST due to contaminant exposure. In this study, levels of hepatic lipidosis, or the lipid accumulation resulting from increased fat mobilization or decreased lipid utilization in injured hepatocytes, were significantly higher in fish from ST than in fish from TP. Glycogen depletion also was more severe in the liver of fish from ST relative to those from TP. Histopathological effects including lipidosis and glycogen deficiency may occur in the liver of fishes in response to contaminant exposure (Hinton, 1994). Severe lipidosis and other histopathological abnormalities occurred in largemouth bass *(Micropterus salmoides)* from a reservoir off the Savannah River, South Carolina, likely in response to PCB contamination (Teh et al., 1997). Moreover, in this study, glycogen depletion was more severe in the liver of fish from contaminated sites relative to those from reference sites.

In contrast to histopathological effects, I did not detect statistically higher levels of CYP1A protein in liver of fish from ST relative to CYP1A protein in fish from other marshes. Higher variability in CYP1A levels within marshes may have been due to differences in sex, age, and sexual maturity of fish sampled. Increased estradiol levels in female fish have been shown to have a suppressive effect on cytochrome P450 protein levels (Buhler and Williams, 1989). Other environmental variables such as salinity,

temperature, suspended sediment levels, and tidal height also may have contributed to increased variability in CYP1A protein levels in the present study.

I demonstrated that the TUNEL assay was an excellent method for evaluating apoptosis as a biomarker response in fish exposed to a wide-range of contaminants, relative to other biomarker methods used in the present study. The TUNEL assay was a sensitive method; apoptotic DNA fragmentation levels were significantly higher in fish from ST, relative to fish from other marshes. In contrast, no differences in levels of CYP1A proteins were found among marshes, and differences in glycogen depletion levels were only detected at P=0.11, indicating that the TUNEL assay was a superior assay, given the environmental conditions of the present study (i.e. contaminant load and types of contaminants). Similar to apoptotic DNA fragmentation, levels of lipidosis and DEVDase activity were higher in fish from ST relative to those in fish from TP. However, there was higher variability in DEVDase activity, lipidosis levels, glycogen depletion, and CYP1A protein levels within treatment groups (i.e. marshes), relative to variability within treatments for apoptotic DNA fragmentation levels. As a result, none of the biomarker responses except apoptotic DNA fragmentation was positively correlated with sediment contaminant levels. In addition to liver biomarker responses, a variety of other biomarker responses and fitness parameters were assessed in extrahepatic tissues of the same fish examined in this study and will be presented in following papers. This will allow for a more rigorous comparison of the sensitivity and reliability of the TUNEL assay relative to multilevel biomarker responses for determining the biological impact of toxicant exposure, and its significance relative to fitness.

The continued application of the TUNEL assay and other biochemical endpoints of apoptosis as biomarkers is central to the field of aquatic toxicology. In contrast to well-known molecular markers such as cytochrome P450 induction and acetylcholinesterase activity, apoptosis is a response that reflects both cellular and tissue-level condition of an organism, and may more accurately indicate organismal stress and fitness. Another advantage of apoptosis over other biomarker responses is that it may occur in response to a diversity of contaminants and thus may be applied to studies of fish from environments ranging in type and magnitude of contamination. This study indicates that the apoptosis is a valuable biomarker response to contaminant exposure. However, future studies are needed to examine temporal fluctuations in background levels of apoptosis in liver and other tissues of fishes held under non-stressful conditions. Moreover, the applicability of the TUNEL and other assays for detecting apoptosis among species, tissues, and over time and the linkages between apoptosis and ecologically relevant effects of toxicant exposure need further exploration.

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Table 1. Average concentrations of selected metals, organic chemicals, and current-use pesticides in sediment from California tidal marshes including China Camp (CC), Carpinteria Salt Marsh (CS), Stege Marsh (ST), Tom's Point (TP), and Walker Creek (WC). ND = not detected; <MDL = below minimum detection limit

| | Marsh (sample size) | | | | |
|---|---|---|---|---|---|
| | **CC (6)** | **CS (36)** | **ST (26)** | **TP (6)** | **WC (6)** |
| **Metals (mg/kg dry wt)** | | | | | |
| Ag | 0.32 | 0.23 | 0.28 | 0.04 | 0.07 |
| Al | 35522.56 | 13649.93 | 31469.58 | 27786.18 | 27648.96 |
| As | 12.27 | 5.10 | 15.90 | 7.37 | 9.74 |
| Cd | 0.24 | 0.23 | 0.46 | 0.18 | 0.21 |
| Cu | 51.30 | 15.07 | 78.33 | 19.16 | 25.63 |
| Pb | 32.93 | 14.28 | 87.94 | 10.32 | 12.64 |
| Se | 0.65 | 0.30 | 0.75 | 0.34 | 0.48 |
| Zn | 109.39 | 81.51 | 272.67 | 67.21 | 68.09 |
| **Organics (μg/kg dry wt)** | | | | | |
| Total PAHs[1] | 1664.0 | 1377.0 | 4271.0 | 927.0 | 960.0 |
| Total PCBs[2] | <MDL | <MDL | 648.0 | <MDL | <MDL |
| p,p'-DDD | 1.4 | 3.7 | 25.0 | ND | ND |
| **Pesticides (μg/kg dry wt)** | | | | | |
| Eptam | ND | ND | 19.9 | ND | ND |
| Pebulate | ND | ND | 4.3 | ND | ND |
| Molinate | ND | ND | 10.9 | ND | ND |
| Cycloate | ND | ND | 3.1 | ND | ND |
| Promethryn | ND | ND | 8.9 | ND | ND |
| Chlorpyrifos | ND | 1.0 | ND | ND | ND |
| Methidathion | ND | 4.1 | 4.6 | ND | ND |
| Napropamide | ND | ND | 5.9 | ND | ND |
| Bifenthrin | ND | 2.3 | ND | ND | ND |
| Permethrin | ND | 3.1 | ND | ND | ND |

[1] Total PAHs = the sum of 33 parent and alkyl substituted PAHs
[2] Total PCBs = the sum of 44 PCB congeners

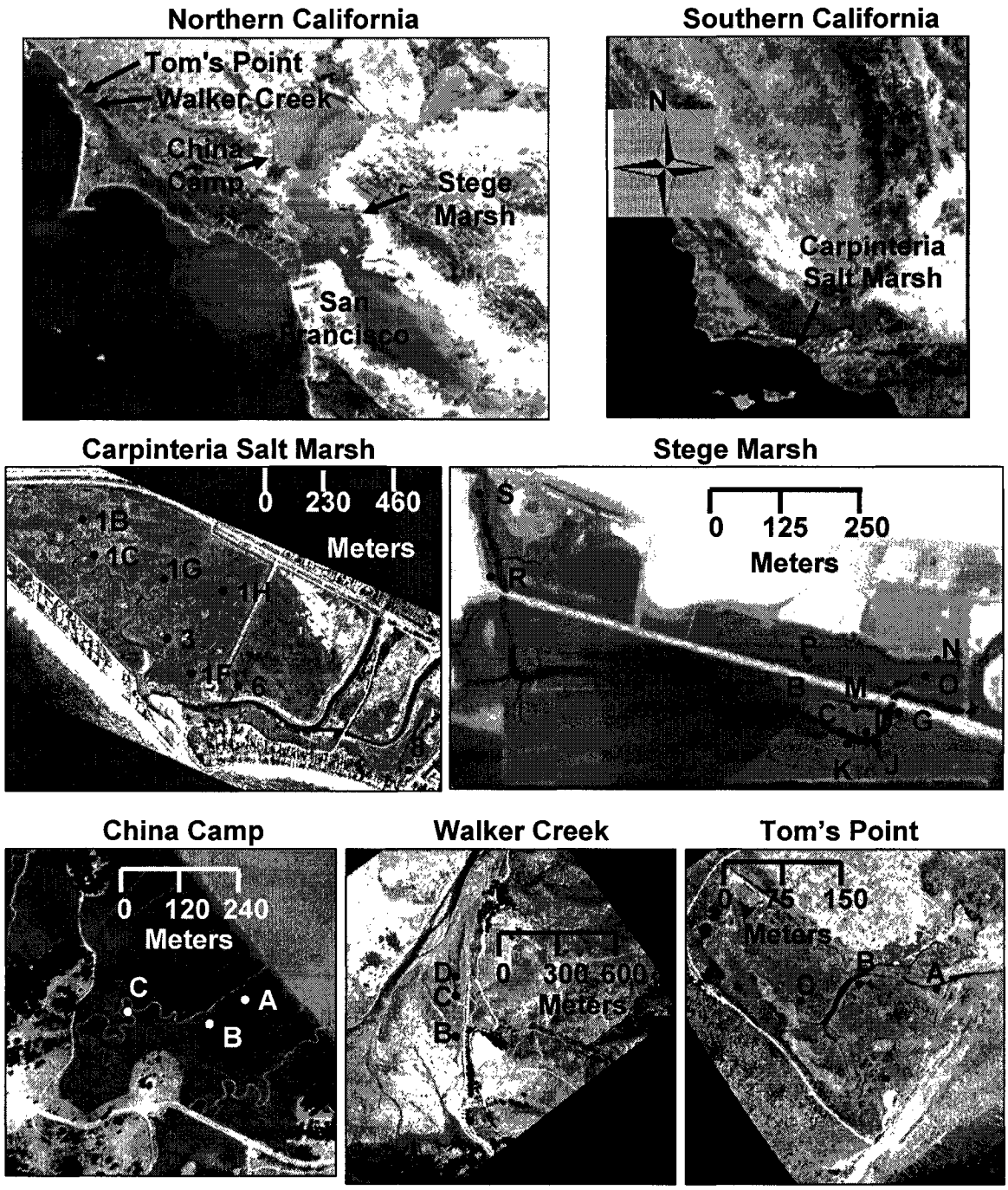


Fig. 1. Map of Northern and Southern California showing the location of marshes sampled in August of 2003 (top), and maps of stations sampled within each California tidal marsh.

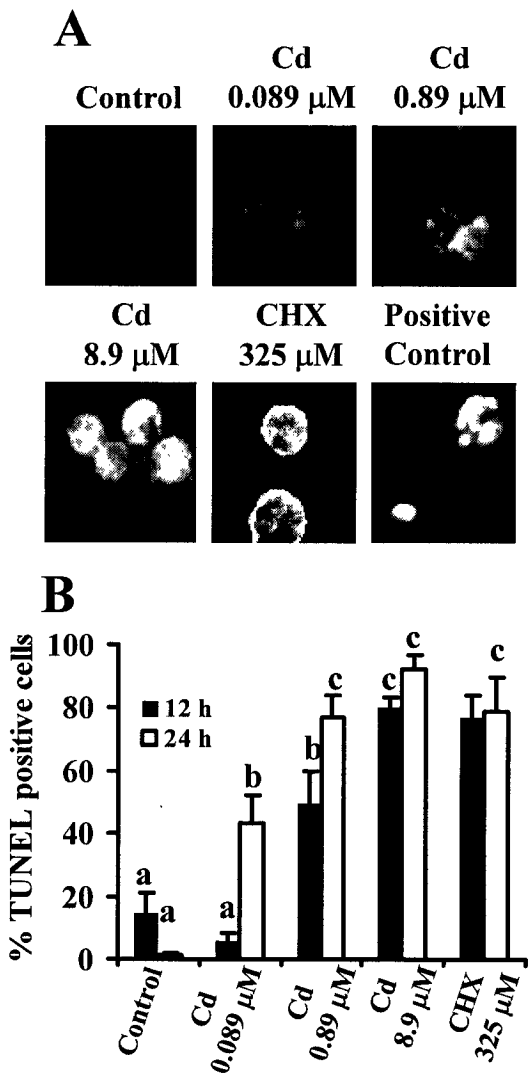


Fig. 2. TUNEL-positive topsmelt hepatocytes demonstrating incorporation of fluorescein-dUTP (A), and percent TUNEL positive topsmelt hepatocytes (+ 1 SE) (B) following exposure to Cd (0 to 8.9 μM) or CHX (325 μM) for 12 or 24 h. Different letters denote significant differences among treatments at $P<0.05$ (n=4/trt).

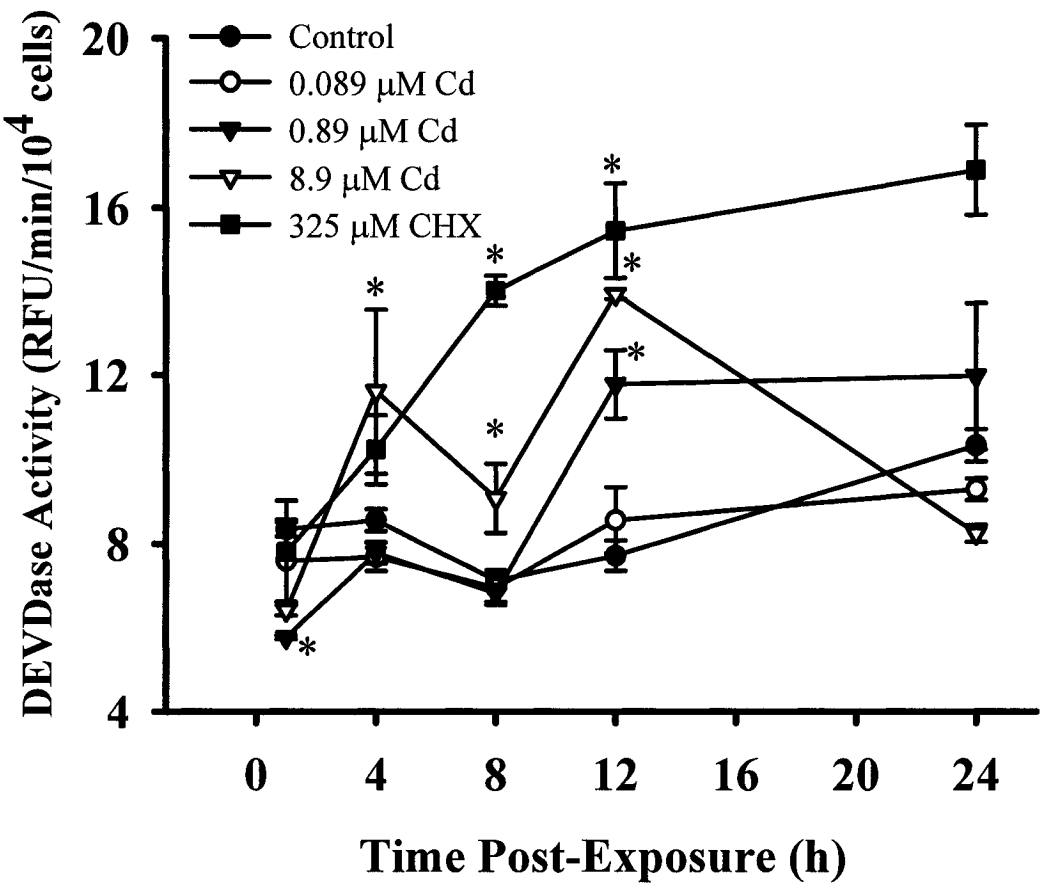


Fig. 3. DEVDase activity (± 1 SE) in topsmelt hepatocytes exposed to Cd (0 to 8.9 μM) or CHX (325 μM) for 1-24 h. DEVDase activity was determined using the Apo-ONE™ Homogeneous Caspase-3/7 Assay. Asterisks denote treatments that are significantly higher than controls at $P<0.05$ (n=4/trt).

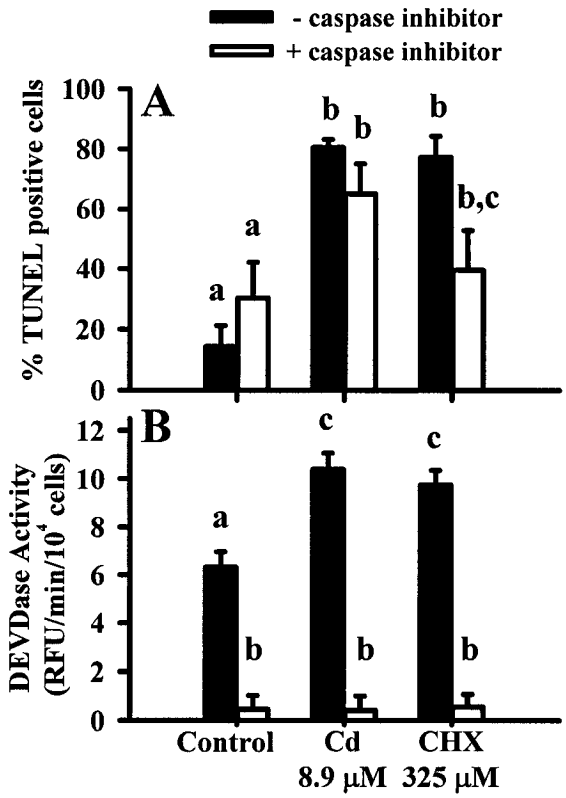


Fig. 4. Percent TUNEL positive cells (A) and DEVDase activity (B) in topsmelt hepatocytes exposed to 0.9% NaCl only (control), Cd (8.9 μM) or CHX (325 μM) in the presence or absence of the pan-caspase inhibitor Z-VAD-FMK (2 μM) for 12 h. Different letters denote significant differences among treatments at $P<0.05$. Error bars represent +1 SE (n=4/trt).

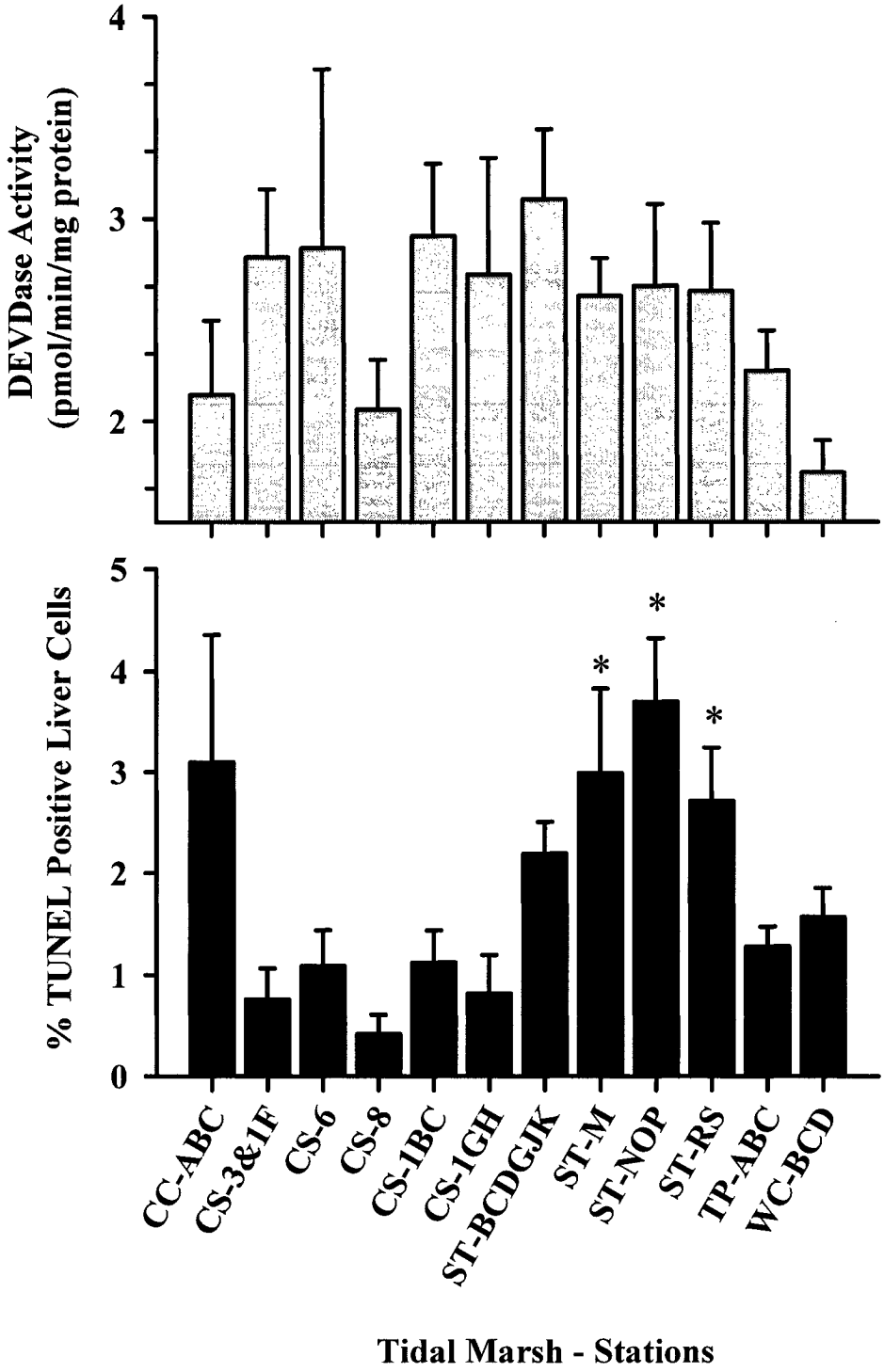


Fig. 5. DEVDase activity and percent of TUNEL positive cells in liver of longjaw mudsuckers collected from combined stations within California tidal marshes in August of 2003. Asterisks indicate significant differences relative to TUNEL positive cells in liver of fish from the reference site, Tom's Point (TP) at $P<0.05$.

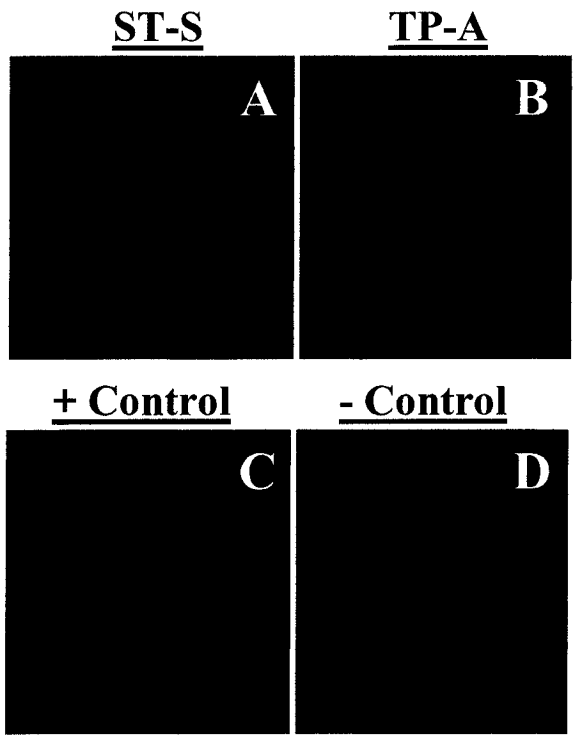


Fig. 6. Fluorescent images of liver cell nuclei of longjaw mudsuckers collected from stations within Stege Marsh (A) and Tom's Point (B). Blue staining is a general cell nucleus stain (Hoecsht 33258), and turquoise staining is indicative of fluorescein-dUTP bound to fragmented DNA. TUNEL assay positive controls were liver sections treated with DNAse (C) and negative controls were processed without the terminal transferase enzyme (D).

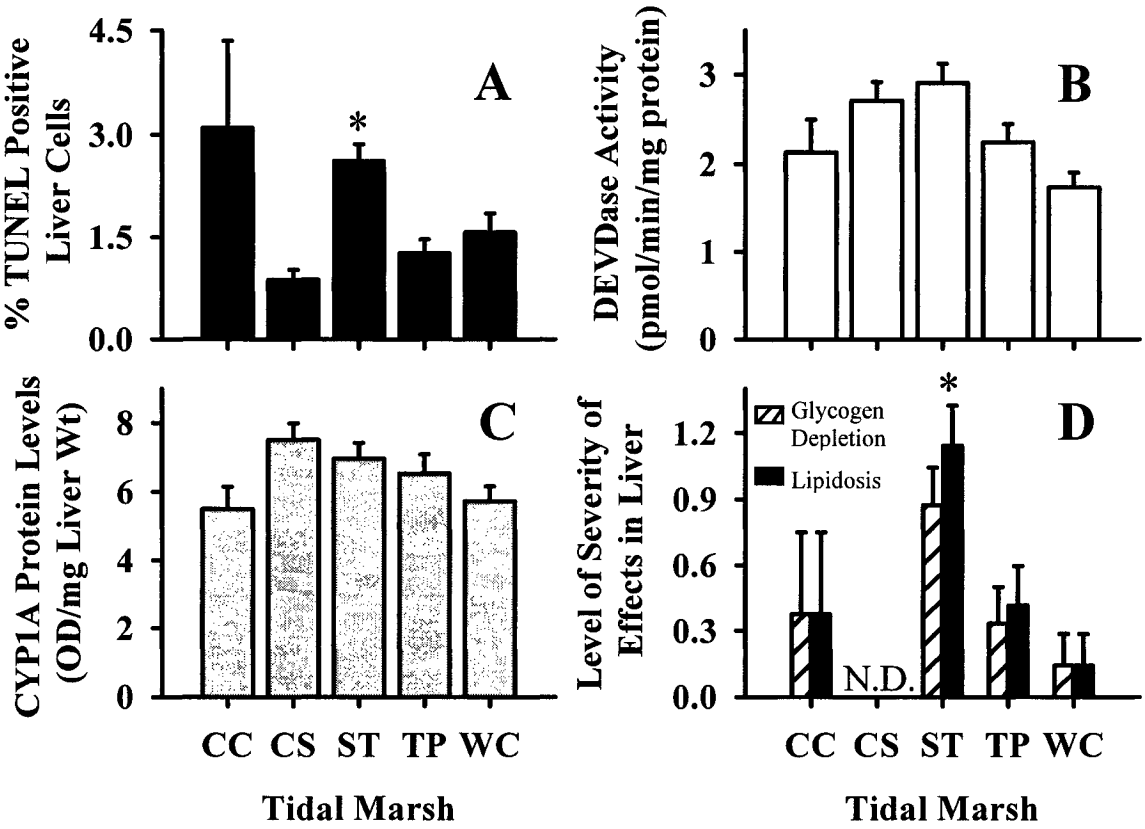


Fig. 7. Mean percent of TUNEL positive cells (+ 1 SE) (A), DEVDase activity (+ 1 SE) (B), CYP1A protein levels (+ 1 SE) (C), and histopathological effects (+ 1 SE) (D) in liver of longjaw mudsuckers collected from various California tidal marshes in August of 2003. Asterisks indicate significant differences relative to fish from the reference site, Tom's Point (TP) at $P<0.05$.

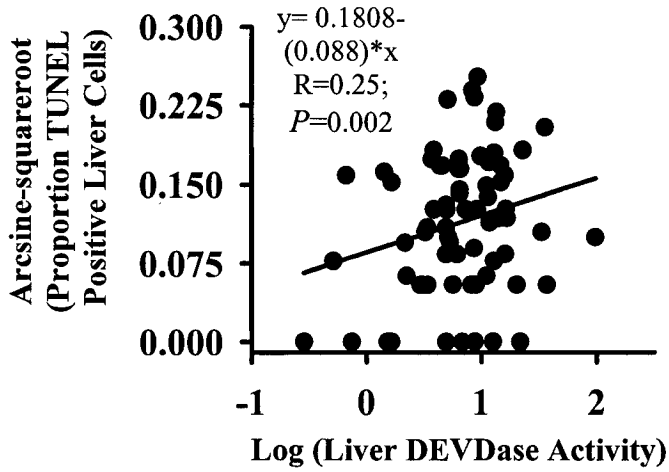


Fig. 8. Proportion of TUNEL positive cells vs. DEVDase activity in liver of mudsuckers sampled in August of 2003 from stations within California tidal marshes.

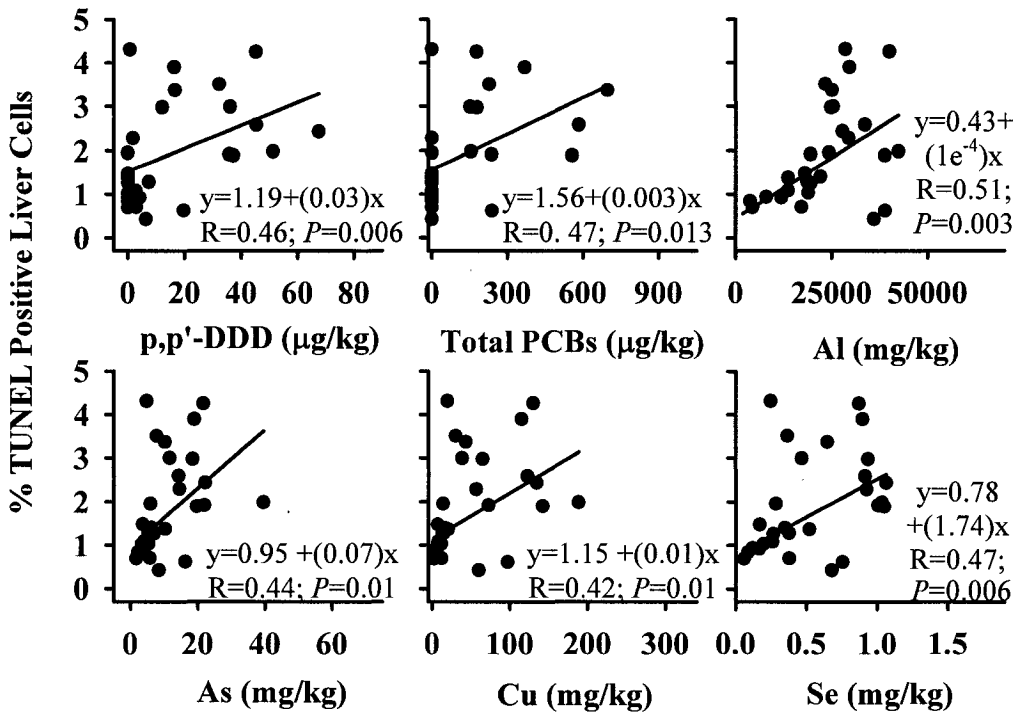


Fig. 9. Mean percent of TUNEL positive cells in liver of longjaw mudsuckers vs. levels of p,p'-DDD, total PCBs, Al, Cu, As, and Se in sediment from stations within California tidal marshes that were sampled in August of 2003. Total PCBs = the sum of 44 PCB congeners.

# CHAPTER 2

## Using an integrated approach to link biomarker responses and physiological stress to growth impairment of cadmium-exposed larval topsmelt

**Abstract**

In this study, I used an integrated approach to determine if key biochemical, cellular, and physiological responses were related to growth impairment of cadmium (Cd)-exposed larval topsmelt (*Atherinops affinis*). Food intake, oxygen consumption rates, apoptotic DNA fragmentation (TUNEL assay), and metallothionein (MT)-like protein levels, were separately measured in relation to growth of larval topsmelt aqueously exposed to sublethal Cd for 14 days. Cadmium accumulation and concentrations of abundant metals were also evaluated in a subset of fish from these experiments. Food intake, weight, and length were inversely correlated with Cd concentration (0 to 100 ppb) and food intake was weakly correlated with final weight of topsmelt exposed to Cd (50 and 100 ppb). Oxygen consumption rates were positively correlated with Cd concentration and mean oxygen consumption rates were inversely correlated with final mean weight of topsmelt. Apoptotic DNA fragmentation and MT-like protein levels were concentration-dependent and were associated with diminished growth of Cd-exposed fish. Apoptotic DNA fragmentation was elevated in the gill of fish exposed to 50 ppb Cd, and in the gut, gill, and liver of fish exposed to 100 ppb Cd. Metallothionein-like protein levels in fish from 100 ppb Cd treatments were significantly higher than those in other treatments. Oxygen consumption rates may have increased as a compensatory response to Cd exposure; however, it is likely that the energy produced was allocated to an increased metabolic demand due to apoptosis, MT synthesis, and changes in ion regulation. This diversion of energy expenditures could contribute to growth impairment of Cd-exposed fish. The integration of biomarker responses across levels of biological organization, as demonstrated here, will improve our ability to use

molecular biomarker responses of organisms to predict population-level consequences of toxicant exposure, and advance our understanding of mechanisms through which fitness becomes impaired.

**Introduction**

Biomarkers, or biological responses of organisms to toxicant exposure, have been used for decades to indicate stress in aquatic organisms and the magnitude of environmental pollution (McCarthy and Shugart, 1990; Handy et al., 2003). Molecular biomarkers such as cytochrome P450 protein induction and activity, acetylcholinesterase activity, and metallothionein induction and protein levels often are evaluated to assess toxicant exposure and effects in aquatic organisms. Ecologically relevant effects such as impaired growth, reduced population success, developmental abnormalities, and changes in population structure also have been measured in response to toxicant exposure (Kurelec, 1993; Anderson et al., 1994; Belfiore and Anderson, 2001). While the relationship between molecular biomarker responses and population level effects of toxicant exposure is often unclear, it has long been suggested that molecular biomarkers should be used in conjunction with measurements of fitness (e.g. growth), endpoints that directly affect the survival of toxicant-exposed organisms and the success of populations (Anderson et al., 1994; Depledge et al., 1995).

There are a few examples of how molecular biomarker responses may be associated with higher level effects of toxicant exposure. Linkages have been drawn between biomarkers of genotoxicity and the initiation and progression of cancer in fishes and mammalian species (Kurelec, 1993; Anderson et al., 1994). Studies also have

correlated biochemical effects of toxicants with fitness. Using vertebrate models such as Atlantic mackerel (*Scomber scombrus*), and *Xenopus laevis* tadpoles, associations were demonstrated between biomarkers of genotoxicity and gross embryonic malformations (Longwell and Hughes, 1980), or growth impairment (Sadinski et al., 1995), respectively. The determination of mechanisms through which toxicant exposure leads to malignant tumor formation is essential. However, ecologically relevant effects may be more prevalent in nature than cancer. Thus, additional research also is necessary to elucidate relationships between molecular biomarker responses and alterations in fitness of toxicant-exposed organisms. Moreover, studies are needed in which biomarker responses are used to advance our understanding of mechanisms through which fitness becomes impaired, instead of only correlating molecular or cellular biomarker responses with an ecologically relevant effect.

More recently, physiologically based measurements such as respiration, metabolism, and digestion have been used as biomarker responses to toxicant exposure. Physiological measurements may provide the key to integrating various molecular and cellular responses in an organism with impaired fitness, because these processes must function properly for an organism to survive (Depledge et al., 1995). A number of studies have demonstrated that toxicant exposure may lead to physiological impairment such as alterations in respiration (Suresh et al., 1993; Beyers et al., 1999; Espina et al., 2000), metabolism (Berntssen and Lundebye, 2001), and feeding (McGeer et al., 2000; Almeida et al., 2002) of fishes. Moreover, growth impairment of fishes has been shown to occur as a result of exposure to a variety of toxicants (Rombaugh and Garside, 1982; Goodman et al., 1992; Marr et al., 1996). However, few studies have integrated toxicant-induced

effects across levels of biological organization from molecular and cellular responses, to physiological effects and fitness impairment. By integrating biomarker responses at multiple levels of biological organization, our ability to use molecular biomarker responses of organisms to predict population-level consequences of toxicant exposure and our understanding of mechanisms through which toxicants impair fitness will be advanced.

In this study, I used an integrated approach to investigate toxicant exposure across multiple levels of biological organization. Because I recently demonstrated that sublethal, subchronic cadmium (Cd) exposure impaired the growth and growth rates of larval topsmelt (*Atherinops affinis*), the present study was designed to examine how changes in key biochemical and cellular biomarker responses and physiological measures were related to growth impairment of Cd-exposed larval topsmelt. Topsmelt was used because it is an EPA-toxicity test species that is found in metal-contaminated tidal marshes in California. My first objective was to determine whether growth impairment of Cd-exposed topsmelt was associated with changes in critical physiological responses (i.e. feeding and respiration). In addition to their ecological relevance, physiological measurements including food intake and respiration are useful because these processes involve energy intake and expenditure, respectively, and thus may be used to quantify the energetic costs of toxicant exposure using mathematical modeling techniques (Nisbet et al., 1997). In a companion paper, the data collected here will be used to generate dynamic energy budget models to explore how Cd changes the rates at which topsmelt acquire and utilize energy for maintenance and growth. My second objective was to determine whether biochemical and cellular effects (i.e. metallothionein levels and apoptotic DNA

fragmentation) of sublethal Cd exposure were associated with growth impairment of topsmelt. I was interested in metallothionein (MT) because it is a low molecular weight metal-binding protein that may be induced following exposure to metals such as Cd, and total MT levels as well as MT induction have long been used as endpoints of Cd exposure in fishes (Olsson, 1993). Apoptosis was also investigated because in contrast to many other biomarkers, apoptosis may directly indicate cellular and organismal damages resulting from metal exposure (Piechotta et al., 1999; Berntssen et al., 2001), and is an endpoint that provides both qualitative (i.e. tissue type) and quantitative (i.e. percent apoptosis) data (Sweet et al., 1999).

Cadmium (Cd) was chosen because it is a heavy metal that contaminates aquatic environments worldwide (Green et al., submitted), and a model contaminant, whose effects have long been investigated in fishes. Cadmium is well-known to impair the growth of fish larvae, the most sensitive life stage to Cd toxicity (Eaton et al., 1978; Rombaugh and Garside, 1982; Woodworth and Pascoe, 1982; Miliou et al., 1998); however, in juvenile and adult fishes, Cd has been shown to cause other physiological effects such as modifications in osmoregulation (McGeer et al., 2000), calcium uptake (Verbost et al., 1987), and reproduction (Spehar, 1976). Within cells, Cd causes numerous biochemical effects and changes in intracellular processes that may lead to the physiological stress observed in Cd-exposed fishes. As a protective response to metal toxicity, Cd induces the synthesis of metallothioneins in target tissues of fishes (i.e. gill, liver, kidney) to sequester toxic Cd ions (Olsson, 1993). However, if not sequestered by metallothioneins or eliminated from the cell, Cd may interfere with processes such as DNA repair and may depress antioxidant levels (e.g. glutathione). These alterations may

lead to DNA and protein damage, the induction of apoptotic and necrotic cell death (Piechotta et al., 1999; Robertson and Orrenius, 2000), and subsequent tissue damage.

## Methods

### *Fish and Water Maintenance*

Eight day post-hatch (dph) larval topsmelt were purchased from Aquatic Bio Systems (Fort Collins, CO) and acclimated under experimental conditions for 24 hr. Water quality (1-μm-filtered seawater at 33 ppth, 20°C, pH = 7.8-8.3, dissolved oxygen > 7 mg/L) and photoperiod (16:8, L:D) were maintained according to topsmelt larval toxicity testing protocols (USEPA, 1995). Fish were fed *Artemia* nauplii *ad libitum* prior to experimentation.

### *Physiological Effects of Cd on Larval Topsmelt*

*Food Intake.* Topsmelt larvae (n=16/trt) were aqueously exposed in a static renewal system (500 mL polypropylene beakers) to seawater alone (200 mL) or to 10-100 μg/L (ppb) Cd in seawater for 14 days. Each test solution was prepared by adding a $CdCl_2$ stock solution to 1-μm-filtered natural seawater from the Bodega Marine Laboratory in Bodega Bay, CA, where the experiments were conducted. Sublethal levels of aqueous Cd exposure in seawater were based on the results of a 7-day mortality study in our laboratory ($LC_{50}$ = 470 ppb). Water changes were performed every other day and water was maintained as described above. Fish were fed 160 *Artemia* nauplii per day and uneaten *Artemia* were removed and counted prior to each feeding. At the end of the experiment, fish were sacrificed on ice, weighed and measured, and a subsample of fish

(n=8/trt) was prepared as described below for evaluation of apoptotic DNA fragmentation by the TUNEL assay. Linear and non- linear regressions were performed to examine the relationships among length, weight, and total *Artemia* eaten by topsmelt vs. Cd concentration (Sokal and Rohlf, 1981). Linear regressions were performed to examine the relationships among weight of topsmelt vs. total *Artemia* eaten by topsmelt in each Cd treatment separately.

*Oxygen Consumption Rates.* Topsmelt larvae (n=20/trt) were aqueously exposed in a static renewal system (500 mL polypropylene beakers) to seawater alone (200 mL) or to 50-250 μg/L (ppb) Cd in seawater for 14 days. I measured oxygen consumption rates in 5 fish per treatment at 1 and 7 day(s) post-exposure, or 10 and 16 dph, respectively. I assessed oxygen consumption rates in 10 fish per treatment at 14 days post-exposure or at age 23 dph. At 10 and 16 dph, sample size was limited to 5 fish per treatment because respiration rate measurements were time-consuming for smaller fish in these age groups relative to those ages 23 dph.

Oxygen consumption rates were measured using a microcathode oxygen electrode (model 1302) with a dissolved oxygen meter (model 781b, Stratkelvin Instruments Ltd., Glasgow, UK). Seawater temperature was maintained at 20+0.05°C by circulating coolant through the outer portion of a jacketed respiration chamber (RC350, Stratkelvin Instruments Ltd., Glasgow, UK). Data was transmitted via a data interface unit to a computer and was analyzed for absolute and normalized oxygen consumption rates using the Stratkelvin 949 Oxygen System (Version 2.2, Stratkelvin Instruments Ltd., Glasgow, UK). Prior to measuring oxygen consumption rates, the electrode was maintained at 20+0.05°C in double deionized water. Topsmelt larvae were starved for 24 h prior to

measuring oxygen consumption rates. For each oxygen consumption rate measurement, one topsmelt larvae was placed in 1.25-1.5 mL of filtered seawater (depending on size) in the respiration chamber and water was stirred using a micro-stir bar and stir plate. Following electrode stabilization, oxygen consumption rate was estimated as the slope of the regression line of oxygen concentration over time at physiologically-relevant concentrations of oxygen (from ~ 7 to 6 mg $O_2$/L for each fish). Background levels of microbial oxygen consumption rates were found to be negligible using 1 μm-filtered seawater with the appropriate concentration of Cd (corresponding to exposure concentrations). Upon completion of oxygen consumption measurements, fish were sacrificed on ice, and weighed and measured.

Linear regression analysis was used to examine the relationship between oxygen consumption rates of larval topsmelt vs. Cd concentration for fish ages 10, 16, and 23 dph, separately (Sokal and Rohlf, 1981). Linear regression analysis also was used to examine the relationship between mean oxygen consumption rates and mean final weight of fish in each Cd treatment. Data was log-transformed and two-way ANOVAs and Student-Newman-Keuls (SNK) multiple comparisons were used to detect differences in mean oxygen consumption rates of topsmelt among Cd treatment levels and among ages.

*Cd Uptake in Larval Topsmelt.* A subsample of topsmelt larvae from the respirometry experiment (n=3/trt) that were exposed to Cd for 14 days were frozen in liquid nitrogen and stored at –80°C until determination of Cd uptake and concentrations of eight abundant metals (K, Na, Mg, Zn, Cu, Sr, Ca, and Fe). Fish were thawed to room temperature (RT) and transferred to a polypropylene tube. Nitric acid was added to a final concentration of 10 μL/mg. After 24 h, the samples were diluted with water to 500

μL/mg. Quantification of Cd and eight abundant metals was by direct aspiration in an ICP-MS (model 7500i, Agilent, Palo Alto, CA) and compared to external standards (traceable to NIST) covering a range from 0.1 μg/L to 20 μg/L. A blank tube was measured as a control, confirming negligible contamination. In the fashion prescribed by EPA method 6020, the instrument detection limit (triple the standard deviation of blanks) was 0.002 μg/L corresponding to a method detection limit of 0.12 μg/L. For each of the elements analyzed, all major isotopes were quantified and all possible interfering elements were determined to ensure negligible interference. No interference corrections were necessary. The instrument was operated with a robust plasma characterized by 0.4% CeO/Ce. Linear regression analyses were used to examine the relationships between Cd concentration and the concentration of eight abundant elements, separately, in whole larval topsmelt vs. Cd concentration in seawater.

*Biochemical and Cellular Effects of Cd Exposure on Larval Topsmelt*

*Apoptotic DNA Fragmentation.* A subsample of topsmelt larvae (n=8/trt) from the growth and food intake experiment described above was fixed in 10% methanol-free formalin for 48 hrs. Samples were rinsed two times in PBS for 10 minutes and held in PBS at 4°C for 1 hr. Samples were transferred to 50% ethanol, held at 4°C for 1 hr, and transferred to 75% ethanol. Samples were further dehydrated, embedded in paraffin, and sectioned according to standard protocols by Central Histology Services (Sacramento, CA). Slides of fish sections were examined for apoptotic DNA fragmentation using the terminal deoxynucleotidyl transferase-mediated dUTP-X nick end labeling (TUNEL) assay.

I used modifications of Gavrieli et al. (1992) and a commercial kit, the Dead-End Fluorometric TUNEL System (Promega, Madison, WI) to examine apoptotic DNA fragmentation in tissues (esophagus, stomach, intestine, liver, kidney, gill) of topsmelt exposed to Cd for 14 days. Briefly, deparaffinized and rehydrated whole body fish sections were incubated with 20 ng/mL proteinase K for 30 min. at 37°C, and rinsed with PBS. Fish sections were incubated with terminal deoxynucleotidyl transferase (TdT) equilibration buffer for 10 min. at RT. The equilibration buffer was replaced with 50 µL of TdT reaction mixture, which contained TdT enzyme, equilibration buffer, and a nucleotide mix with fluorescein-12-dUTP, and incubated at 37°C for 1 h. The reaction was stopped by immersing the slides in 2X SSC for 15 min., and the slides were rinsed three times in fresh PBS containing 0.1% Triton ® X-100 and 5mg/mL of BSA, and one time in PBS alone. The slides were then counterstained with Hoechst 33258 solution (Molecular Probes, Inc., Eugene, OR) freshly diluted to 0.1µg/mL in PBS for 15 min. at RT in the dark. After rinsing in double deionized water, slides were air dried and mounted in a medium containing 90% glycerol, 10% PBS, 0.2% n-propyl gallate. Slides were stored at -20°C for 1-2 days until further analysis. An Olympus BX50WI microscope (Olympus America, Melville, NY) with a standard fluorescein filter set was used to view the green fluorescence of TUNEL positive nuclei and the blue fluorescence of the Hoechst stain, which stains all cell nuclei, was detected using a DAPI/Hoechst filter at approximately 460 nm. Images of all nuclei (blue fluorescence) were overlaid with images of TUNEL positive cells in each field to determine the percent of cell nuclei that were TUNEL positive. Data was arcsinesquareroot transformed when necessary and one-way fixed-factor ANOVAs and SNK multiple comparisons were used to detect

differences in the proportions of TUNEL positive cells among Cd treatments for each tissue separately (Sokal and Rohlf, 1981). Pearson Product Moment Correlations were performed to evaluate the relationship between TUNEL positive cells in the gill of topsmelt and final wet weight of topsmelt exposed to Cd for 14 days.

*Metallothionein Levels*. Topsmelt larvae (n=24/trt) were aqueously exposed to Cd as described above. At the end of the experiment, fish were sacrificed on ice, and weighed and measured. Fish were frozen in liquid nitrogen, and stored at –80°C until analysis. Metallothionein-like protein levels were examined in three pools of 7-8 fish/trt.

Pooled fish samples were analyzed for MT-like protein levels using the monobromobimane derivatization method of Fan et al. (2004). Briefly, pooled samples were freeze-dried for 24 h and were homogenized in liquid nitrogen using mortar and pestle. Samples were weighed and dissolved in HEPPS buffer (50 mM HEPPS + 5 mM DTPA in 50% acetonitrile, pH 8) with 1 μmol DTT for 20 min. at 70°C. Samples were weighed and incubated in HEPPS buffer (50 mM HEPPS + 5 mM DTPA in 50% acetonitrile, pH 8) with 1 μmol DTT for 20 min. at 70°C, followed by derivatization with monobromobimane (44.7 mM) for 30 min. at 70°C in the dark. After samples had cooled to 4°C, samples were centrifuged at approximately 13,000 rpm for 10 min. The tissue pellet was discarded and monobromobimane-derivatized proteins including MT in the supernatant were precipitated with 80% acetonitrile, centrifuged, and washed 3 times in 80% acetonitrile. The protein pellet was dried under vacuum, dissolved in BioRad Tricine-SDS sample buffer, denatured at 95°C, and stored at -80°C until electrophoresis. Samples and rabbit metallothionein standards were electrophoresed at 100V for 1.5 h in a tricine-SDS polyacrylamide gel. Images of monobromobimane-derivatized proteins on

gels were captured under UV black light using digital photography and MT-like protein bands were quantified using ImageJ software (National Institutes of Health, USA). One-way fixed-factor ANOVAs and SNK multiple comparisons were used to detect differences in the MT-like protein levels among Cd treatment groups (Sokal and Rohlf, 1981). Pearson Product Moment Correlations were performed to evaluate the relationship between mean MT-like protein levels and mean final weight of topsmelt.

*Cd Chemistry*

In each experiment separately, water samples were randomly taken from two beakers at each Cd concentration level both before and after one water change, for a total of n=4/trt. Because of a cold room failure, the first samples taken during the respirometry experiment were below nominal Cd concentrations, presumably because Cd formed an insoluble precipitate, such as a sulfide. Thus, additional samples were taken and Cd concentrations verified. All samples were stored in the dark at 4°C and analyzed within a few days after sampling. Samples were brought to room temperature, gently stirred to return condensed vapor to the solution in the bottom of the tube. Samples (250 μL) were diluted 20-fold in 1% nitric acid (Trace Metal grade, Fisher Scientific, Pittsburgh, PA) in a new polypropylene centrifuge tube. A blank tube was measured as a control, confirming negligible contamination. Quantification was by direct aspiration in an ICP-MS (model 7500i, Agilent, Palo Alto, CA) and compared to external standards (traceable to NIST) covering a range from 0.1 μg/L to 20 μg/L. In the fashion prescribed by EPA method 6020, the instrument detection limit (triple the standard deviation of blanks) was 0.006 μg/L corresponding to a method detection limit of 0.12 μg/L. Both Cd isotopes 111

and 114 were measured, and elements Mo and Sn were monitored to ensure negligible interference. The instrument was operated with a robust plasma characterized by 0.4% CeO/Ce.

## Results

*Physiological Effects of Cd on Larval Topsmelt*

Length, weight, and food intake (*Artemia*) of larval topsmelt were negatively correlated with Cd concentration (Table 2, Fig. 10). Cd concentration explained more of the variability in weight and length than in food intake, or total *Artemia* eaten during the 14 day exposure. In the 50 and 100 ppb Cd treatments, weight was correlated with total *Artemia* eaten at $P<0.05$ (Fig. 11). Fish in control treatments demonstrated marginally significant correlations between weight and total *Artemia* eaten, while the correlation between weight and *Artemia* eaten in fish from 10 ppb Cd treatments was not significant at $P<0.05$.

Oxygen consumption rates of larval topsmelt varied among Cd treatments and age groups (Two-way ANOVA, $P<0.05$). At ages 10 and 23 dph, oxygen consumption rates were positively correlated with Cd concentration, while at age 16 dph, oxygen consumption rates were not significantly correlated with Cd concentration (Table 3, Fig. 12). However, regardless of age, fish exposed to 250 ppb Cd demonstrated significantly higher oxygen consumption rates than those of controls ($P=0.02$). Mean oxygen consumption rates of larval topsmelt were significantly correlated with final mean weight of fish exposed to Cd for 14 days (Fig. 13, $P=0.002$).

The Cd concentration that accumulated in larval topsmelt exposed to Cd (0-250 ppb) for 14 days was positively correlated with increasing Cd concentration in seawater (Fig. 14). The Ca content of larval topsmelt exposed to Cd was negatively correlated with increasing Cd concentration in seawater (Fig. 14). The Cu concentration in larval topsmelt exposed to Cd for 14 days also was negatively correlated with Cd concentration in seawater at $P=0.04$ ($r^2=0.16$). The concentrations of other abundant metals analyzed in larval topsmelt including K, Na, Mg, Zn, Sr, and Fe demonstrated slight, but non-significant negative correlations at $P<0.05$ with increasing Cd concentration in seawater.

*Biochemical and Cellular Effects of Cd on Larval Topsmelt*

The TUNEL assay was used to assess apoptotic DNA fragmentation, measured as TUNEL positive cells, in various tissues of topsmelt. I detected higher levels of TUNEL positive cells in the gut (i.e. intestine and stomach) and liver of topsmelt exposed to 100 ppb Cd relative to those of controls (ANOVA, $P<0.05$, Figs. 15,16A). Fish exposed to 50 and 100 ppb Cd demonstrated higher levels of TUNEL positive cells in gills relative to those of control fish (ANOVA, $P<0.01$). Differences in the percent TUNEL positive cells in kidneys were marginal among Cd treatments (ANOVA, $P=0.051$), but no differences in the percent of TUNEL positive cells in intestine were found among Cd treatments (ANOVA, $P=0.461$). Elevated levels of TUNEL positive cells in the gut, gill, and liver were found in a subset of fish whose final mean weight, or growth was diminished at the highest Cd concentrations relative to controls (Fig. 10). A negative correlation was found between the final wet weight vs. the proportion of TUNEL positive cells in the gill of topsmelt exposed to Cd for 14 days (Fig. 16B). The final wet weight was not correlated at

$P<0.05$ with the proportion of TUNEL positive cells in the gut, or liver, separately, of larval topsmelt exposed to Cd (0-100 ppb).

Topsmelt exposed to 100 ppb Cd for 14 days demonstrated higher levels of MT-like proteins relative to controls (Fig. 17), and levels were significantly higher than those of fish in all other Cd treatments (ANOVA, $P<0.001$). While mean MT-like protein levels were not significantly correlated with final mean weight of topsmelt at $P<0.05$, elevated MT-like protein levels were detected in fish exposed to 100 ppb Cd, a concentration that also led to reduced growth rates in the same group of fish (ANOVA, $P<0.05$).

*Cd Chemistry*

In the food intake experiment, mean Cd concentrations ($\pm$ 1 SD) that corresponded to 0, 10, 50 and 100 ppb nominal Cd concentrations randomly sampled from four beakers per treatment were as follows: <0.1, 10.5 $\pm$ 2.7, 52.8 $\pm$ 3.2, and 118 $\pm$ 4.5 ppb. In the respirometry experiment, mean Cd concentrations ($\pm$ 1 SD) that corresponded to 0, 50, 100, and 250 ppb nominal Cd concentrations were sampled from four beakers per treatment were as follows: 0.25 $\pm$ 0.5, 46.6 $\pm$ 2.5, 89.3 $\pm$ 0.3 and 220.8 $\pm$ 5.0 ppb. In the metallothionein experiment, mean Cd concentrations ($\pm$ 1 SD) that corresponded to 0, 10, 50 and 100 ppb nominal Cd concentrations sampled from four beakers per treatment were as follows: 0.06 $\pm$ 0.02, 9.1 $\pm$ 0.3, 44.8 $\pm$ 1.4, and 83.8 $\pm$ 6.0 ppb. Cd concentrations analyzed during the food intake experiment were the same as Cd concentrations for the apoptotic DNA fragmentation experiment because subsamples of fish from the food intake experiment were analyzed for apoptotic DNA fragmentation.

**Discussion**

To my knowledge, this is the first study to demonstrate that biochemical, cellular, and physiological effects of Cd exposure were related to growth reductions of larval fish. Food intake, weight, and length were inversely correlated with Cd concentration and food intake was positively correlated with final weight of topsmelt exposed to Cd (50 and 100 ppb). Oxygen consumption rates were positively correlated with Cd concentration and mean oxygen consumption rates were inversely correlated with final mean weight of topsmelt. I found tissue- and concentration-specific differences in apoptotic DNA fragmentation in fish exposed to Cd, and elevated levels of DNA fragmentation were associated with diminished growth of Cd-exposed fish. In fish exposed to 50 ppb Cd, apoptotic DNA fragmentation was elevated in the gill relative to controls, whereas DNA fragmentation was elevated in the gut, gill, and liver of fish exposed to 100 ppb Cd. Metallothionein-like protein levels in fish from 100 ppb Cd treatments were significantly higher than those in other treatments and corresponded to reduced growth of fish from this Cd treatment level. The elevated oxygen consumption rates and increased levels of apoptotic DNA fragmentation may explain growth reductions in fish exposed to Cd.

*Physiological Effects of Cd on Larval Topsmelt*

In this study, weight, length, and food intake of topsmelt decreased significantly with increasing Cd concentration, suggesting that reductions in energy or food intake contributed to growth impairment of Cd-exposed topsmelt. Similar to my findings, many studies have demonstrated that sublethal Cd exposure causes growth impairment of

fishes. Cadmium exposure led to diminished growth of larval or juvenile brook trout (*Salvelinus fontinalis*), Atlantic salmon (*Salmo salar*), guppies (*Poecilia reticulata*), and rainbow trout (*Salmo gairdneri*) (Eaton et al., 1978; Rombaugh and Garside, 1982; Woodworth and Pascoe, 1982; Miliou et al., 1998); however, in these investigations physiological responses contributing to growth impairment were not examined. In contrast, while reductions in food intake and digestibility were observed in Cd-exposed juvenile and adult fishes, growth reductions were not detected (McGeer et al., 2000; Berntssen and Lundebye, 2001; Almeida et al., 2002). In the present study, correlations were stronger between food intake and weight of fish exposed to the highest Cd concentrations, as compared to food intake and weight relationships of control fish. However, only some of the variability in weight may be explained by food intake for fish exposed to Cd (50 and 100 ppb), indicating that food intake was not the only factor contributing to growth impairment of Cd-exposed topsmelt.

Oxygen consumption rates of topsmelt at ages 10 and 23 dph (1 and 14 days post-exposure) increased with increasing Cd concentrations, suggesting that topsmelt may compensate for the metabolic costs associated with Cd exposure through increases in oxygen uptake. A shift in the whole fish metabolic rate, measured as oxygen consumption rate, is well known to occur as an adaptive response to extreme environmental conditions or toxicant exposure (Brett and Groves, 1979; Marr et al., 1996). Specifically, an increase in metabolic rate provides more oxygen for aerobic conversion of the energy contained in food to the chemical energy stored as ATP that is necessary for repair and maintenance purposes. Thus, in the present study, it is likely that the elevated oxygen consumption rates of Cd-exposed topsmelt reflected the need for

increased oxidative metabolism and ATP production for protein synthesis (e.g. metallothioneins), detoxification, or metal elimination processes. Similar to my findings, sublethal Cd exposure led to increased oxygen consumption rates as well as oxygen extraction efficiency of juvenile grass carp (*Ctenopharyngodon idella*) (Espina et al., 2000). Moreover, elevated oxygen consumption rates in fry of the common carp (*Cyprinus carpio*) exposed to sublethal Cd concentrations were associated with increases in succinate dehydrogenase, an enzyme important in oxidative metabolism (Suresh et al., 1993). It is also possible that oxygen consumption rates increased because of hyperactivity induced by Cd exposure. Locomotor activity significantly increased in bluegill (*Lepomis macrochirus*) (Ellgaard et al., 1978), and gill ventilatory rates increased in rainbow trout exposed to sublethal concentrations of Cd (Majewski and Giles, 1981). Alternatively, increases in oxygen consumption rates may be the result of a general stress response of topsmelt to Cd exposure. In juvenile and adult fishes, studies have demonstrated that sublethal Cd exposure is stressful, as measured by cortisol increases (Ricard et al., 1998), and glycogen depletion in the liver, (Soegas et al., 1996) and muscle (Almeida et al., 2002).

While oxygen consumption rates of fish age 16 dph (7 days post-exposure) were significantly higher for fish in 250 ppb Cd treatments relative to controls, there was no effect of Cd on oxygen consumption rates of fish exposed to moderate Cd concentrations (50 and 100 ppb). For fish age 16 dph, sample size was limited to five fish per treatment because respiration rate measurements were time-consuming for fish in this age group relative to measurements of fish at age 23 dph (n=10/trt). Thus, my inability to detect small increases in oxygen consumption rates at these Cd concentrations may be due to a

small sample size and low power to measure increases in oxygen consumption rates. Alternatively, increases in mean oxygen consumption rates of Cd-exposed fish may have been masked by artificial increases in baseline oxygen consumption rates resulting from confinement stress during respirometry measurements (Cech, 1990).

I found that higher oxygen consumption rates of Cd-exposed topsmelt were associated with reduced growth, or final mean weight of fish, indicating that less energy was allocated for growth because of an increased metabolic demand for detoxification or elimination of Cd. While it is well known that growth may be limited as a result of increases in metabolic rate (Brett and Groves, 1979), no studies to date have examined the relationship between energetic costs of Cd exposure and growth reductions in fish exposed to waterborne Cd. These relationships have been examined in fish exposed to other toxicants. Similar to my findings, oxygen consumption rates were elevated, food intake reduced, and growth diminished in juvenile large-mouth bass *(Micropterus salmoides)* exposed to dieldrin for 16 days (Beyers et al., 1999), indicating that reduced growth may have occurred as a result of reduced energy intake and increased energy expenditures. My findings and those of Beyers et al. (1999) are of interest because physiological measurements including food intake, respiration, and growth, are processes that may be used to quantify the energetic costs of toxicant exposure using mathematical modeling techniques. The application of modeling to toxicological investigations allows for the determination of the variables that are primarily responsible for growth impairment of fishes. Thus, in a following paper dynamic energy budget models will be generated using data presented here, to determine how Cd changes the rates at which topsmelt acquire and utilize energy for maintenance and growth. My results may have

important consequences for population dynamics because contaminant-induced alterations in the energy fluxes within individuals have been shown to affect the persistence and stability of populations, using modeling techniques (Nisbet et al., 1997).

The accumulation of Cd and reduction in Ca concentrations in topsmelt exposed to waterborne Cd suggest that ion regulation was altered in Cd-exposed topsmelt. Cadmium is well-known to interfere with ion regulation in freshwater fish through the inhibition of Ca influx into the gills (Verbost et al., 1987). In marine species, which cope with an osmotic loss of water, the disruption of ion regulation may be equally important in the intestine and kidneys; Ca uptake may be suppressed by Cd in tissues such as intestine through the inhibition of the Na/Ca exchanger protein (Schoenmakers et al., 1992). Fish exposed to metals may compensate for the disruption of ion regulation by increasing the number of chloride cells in the gills (Verbost et al., 1987; McGeer et al., 2000). Because compensatory mechanisms used to regain homeostasis in organisms are energetically costly, it is possible that reductions in growth of Cd-exposed topsmelt in the present study were indirectly related to changes in water and ion balance.

*Biochemical and Cellular Effects of Cd on Larval Topsmelt*

Apoptotic DNA fragmentation, measured as the percent of TUNEL positive cells, was elevated in Cd-exposed topsmelt relative to controls, but was dependent on tissue and Cd concentration. In fish exposed to 50 ppb Cd, apoptotic DNA fragmentation was elevated in the gill relative to controls, indicating that Cd was causing toxic effects in the gills. In contrast, apoptotic DNA fragmentation in fish exposed to 100 ppb Cd was elevated in the gut, gill, and liver, tissues that are well-known to accumulate Cd (Eisler,

1974), and thus susceptible to Cd toxicity. Cadmium has been shown to induce DNA fragmentation in liver of dab (*Limanda limanda*) (Piechotta et al., 1999), and apoptotic cell death in the intestine of Atlantic salmon parr exposed to dietary Cd (Berntssen et al., 2001); however, to my knowledge, apoptosis has not been examined in gill and gut of fish exposed to waterborne Cd. It was not surprising that DNA fragmentation occurred in the gill of Cd-exposed topsmelt because the teleost gill epithelium is a major site of gas exchange and ion regulation, and waterborne Cd may be readily taken up by branchial epithelial cells of the fish gill (Verbost et al., 1987). In addition, metals including Cd, copper, and zinc have been shown to cause structural and functional damage to fish through aqueous metal exposures. In contrast to gills, apoptotic DNA fragmentation in the gut of Cd-exposed topsmelt may have occurred because marine teleosts continuously drink water as part of their osmoregulatory strategy (Bond, 1996). The present study differs from previous investigations of metal-induced apoptosis in fishes because I demonstrated that Cd-induced apoptotic DNA fragmentation in topsmelt was accompanied by impaired growth. Furthermore, I measured significant correlations between apoptotic DNA fragmentation in the gill and reduced growth of topsmelt. Energy in the form of ATP is required for cells to undergo apoptosis, a highly controlled form of cell death (Robertson and Orrenius, 2000). Thus, the increased levels of apoptosis found in Cd-exposed fish in my study may have contributed to an increased metabolic demand, and subsequent reductions in growth.

The level of MT-like proteins were higher in fish exposed to 100 ppb Cd than in fish from all other treatment groups, suggesting an induction of this protein by exposure to 100 ppb Cd. Cadmium exposure is well-known to induce MT synthesis in fishes

(Olsson, 1993). Metallothioneins are important for the protection of cells against Cd toxicity because they are known to sequester Cd, and increase metal tolerance. While I did not measure tissue-specific levels of the MT-like protein in this study, the elevated levels of apoptotic DNA fragmentation in gill, gut, and liver of topsmelt exposed to 100 ppb Cd suggest that the MT-like protein in these tissues could not have sequestered all of the Cd present. Berntssen et al. (2001) demonstrated that MT bound only a small proportion of the Cd in the kidney of Atlantic salmon. Moreover, MT proteins bound only some of the cytosolic Cd in the liver of Cd-exposed sea bass *Dicentrarchus labrax* (Cattani et al., 1996).

High MT-like protein levels were associated with reductions in growth of fish exposed to 100 ppb Cd, suggesting that there are metabolic costs associated with the synthesis of this protein or detoxification processes in Cd-exposed topsmelt. Similar to my findings, Wu and Hwang (2000) demonstrated an increase in MT protein levels that was associated with growth impairment of Cd-exposed tilapia (*Oreochromis niloticus*) larvae. More specific evidence of the energetic costs of MT synthesis were demonstrated by Cattani et al. (1996), who found a significant relationship between increased MT levels and decreased adenylate energy charge in Cd-exposed sea bass.

The MT-like protein levels in fish exposed to 50 ppb Cd were not significantly higher than those in control fish. However, the diminished growth, elevated oxygen consumption rates, and increased levels of apoptotic DNA fragmentation in the gill of fish exposed to 50 ppb Cd, provide evidence that Cd was exerting its toxic effects primarily on the gill of fish at this concentration. Exposure to high levels of heavy metals including Cd is well known to affect the gill structure and function. Acute exposure to

lethal Cd concentrations led to hypertrophy of gill filaments, and hyperplasia and necrosis of the respiratory gill lamellae in the killifish *Fundulus heteroclitus*, and increased mucus secretion in many species, both of which have been associated with depressed respiratory function of the gill (Gardner and Yevich, 1979; Evans, 1987; Verbost et al., 1987). Because sublethal concentrations of Cd were used in my study, topsmelt were likely able to compensate for the minor gill damage (i.e. apoptosis) by increasing their metabolic rates through increased oxygen consumption. The lack of increases in MT-like protein levels in fish exposed to 50 ppb Cd may be explained by the fact that MT mRNA expression and protein levels in gills may not rise until more than 14 days post-exposure (De Smet et al., 2001; Lange et al., 2002), the duration of Cd exposure in my study. It is also likely that MTs in gills are less capable of sequestering Cd than liver and kidney MTs (Lange et al., 2002). Alternatively, the lack of increases in MT-like protein levels of fish exposed to 50 ppb Cd may be due to the limitations of measuring MT in pooled samples of fish larvae (Marr et al., 1996); tissue-specific increases in MT levels may have been obscured by high levels of variability in constituent MT levels among tissues and among fish within each pooled sample.

*Conclusions*

I used an integrated approach to investigate toxicant exposure across multiple levels of biological organization by measuring biochemical, cellular, and physiological biomarker responses in conjunction with the growth of Cd-exposed larval topsmelt. This approach allowed me to evaluate the sensitivity of biomarker responses for measuring Cd toxicity and determine whether any of the biomarkers would be useful in predicting

population-level effects, based on their linkage to fitness (i.e. growth) impairment. Apoptotic DNA fragmentation appeared to be the most informative and sensitive endpoint of Cd toxicity in larval topsmelt. I discerned tissue- and concentration-dependent differences in apoptotic DNA fragmentation, measured as TUNEL positive cells, and apoptotic DNA fragmentation was associated with diminished growth of Cd-exposed fish, indicating its potential usefulness as an early warning indicator of significant adverse effects. Metallothionein-like protein levels in whole fish larvae were a less useful response because increases in these protein levels were found at higher Cd concentrations (100 ppb) than the lowest concentration of Cd (50 ppb) that caused diminished growth. However, in larger fish, where tissue-specific differences in MT-like proteins could be discerned, it is likely that MT would be a more sensitive biomarker of Cd exposure than it was in the present study. Food intake also was not informative because much of the variability in growth could not be explained by food intake, whereas oxygen consumption rates, or respiration, of the fish, was related to fish growth and thus provided insight into the mechanisms of Cd-impaired growth of topsmelt.

The assessment of biomarkers from multiple levels of biological organization in this study improved our understanding of the mechanisms through which growth is impaired in Cd-exposed fish. Nonetheless, future studies should be performed to evaluate how each biomarker response changes over time and to determine whether this combination of biomarkers may be useful for predicting fitness impairment in fishes from contaminated environments.

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Table 2. Results of non-linear ($y=a*x+bx^2$) regression analyses between weight, length, or total *Artemia* eaten, and log Cd concentration.

| **Regression** | **df** | **MS** | **F** | **P** | **$r^2$** |
|---|---|---|---|---|---|
| (A) Weight vs. log [Cd] | 2 | 19.0 | 5.52 | 0.006 | 0.13 |
| Error | 59 | 3.4 | | | |
| (B) Length vs. log [Cd] | 2 | 5.6 | 7.4 | 0.001 | 0.17 |
| Error | 59 | 0.8 | | | |
| (C) Total Artemia Eaten vs. log [Cd] | 2 | 96442 | 3.36 | 0.04 | 0.07 |
| Error | 59 | 28679 | | | |

Table 3. Results of linear regression analyses between oxygen consumption rates of larval topsmelt vs. log Cd concentration at ages 10, 16, and 23 dph, or 1, 7, and 14 days post-exposure, respectively.

| Regression | df | Coefficient | Std Error | t | P | $r^2$ |
|---|---|---|---|---|---|---|
| (A) Oxygen Consumption at 10 | 1 | 0.18 | 0.086 | 2.11 | 0.04 | 0.15 |
| (B) Oxygen Consumption at 16 | 1 | -0.006 | 0.085 | -0.07 | 0.95 | 0.00 |
| (C) Oxygen Consumption at 23 | 1 | 0.15 | 0.060 | 2.56 | 0.02 | 0.15 |

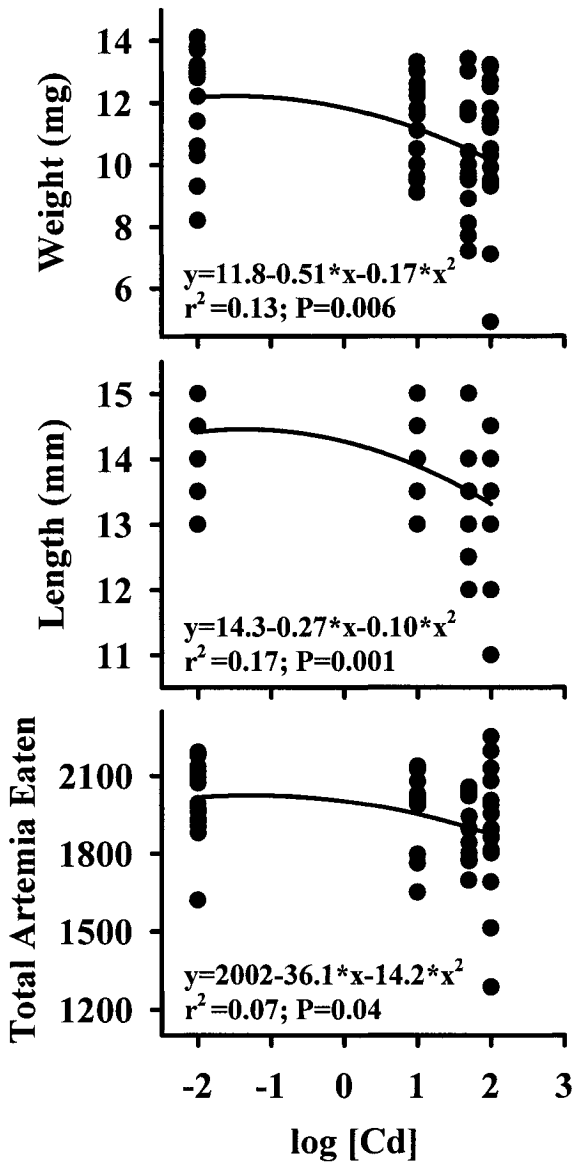


Fig. 10. Final mean weight, final mean length, and total *Artemia* eaten by topsmelt vs. Cd concentration (0-100 ppb) to which topsmelt were exposed for 14 days.

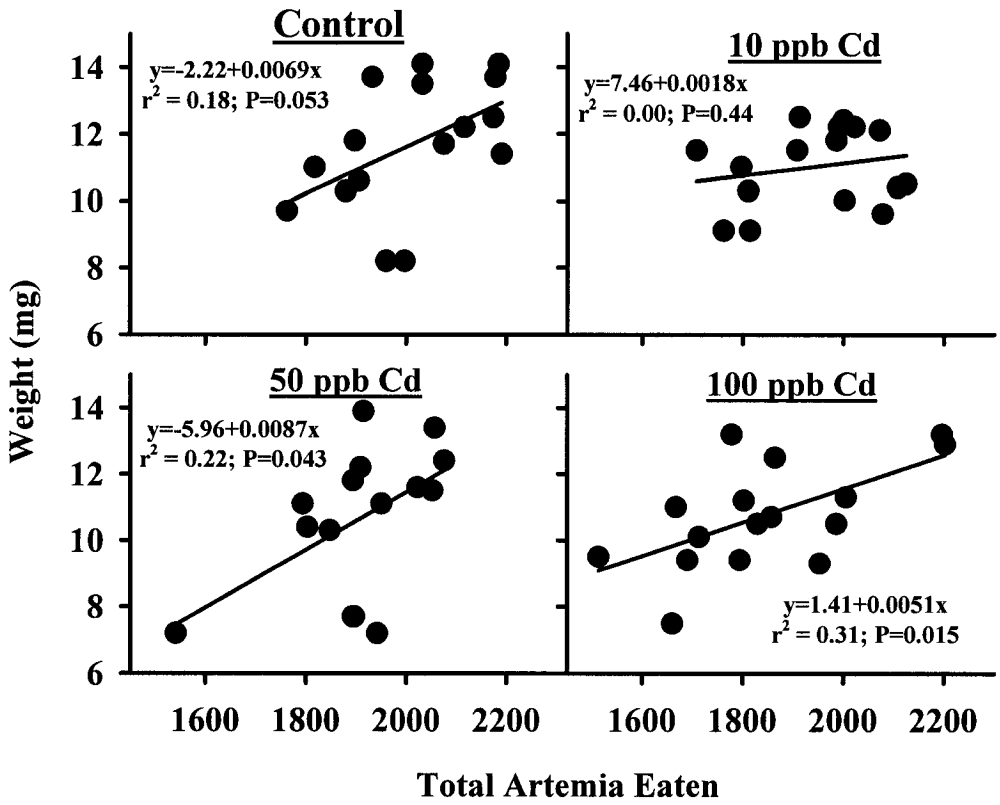


Fig. 11. Weight of larval topsmelt vs. total *Artemia* eaten during 14-day exposures to seawater alone (control) or Cd (10 to 100 ppb).

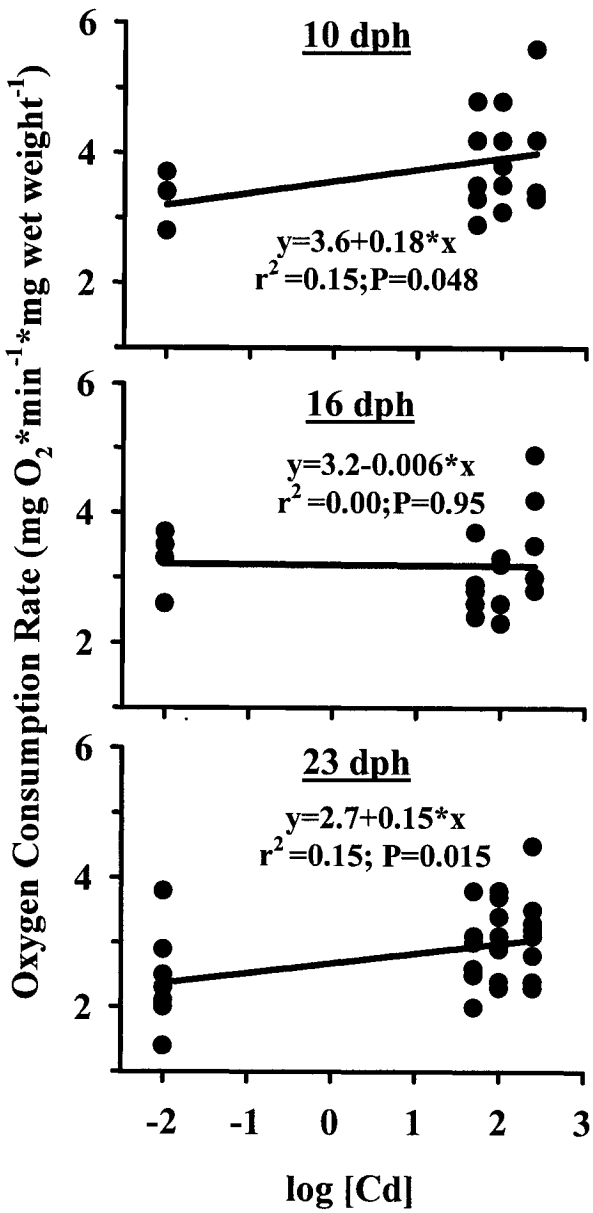


Fig. 12. Oxygen consumption rates (± 1 SE) of larval topsmelt vs. Cd concentration (0-250 ppb) to which topsmelt were exposed for 1 to 14 days. Oxygen consumption rates were measured at ages 10, 16, and 23 dph, or at 1, 7 and 14 days post-exposure.

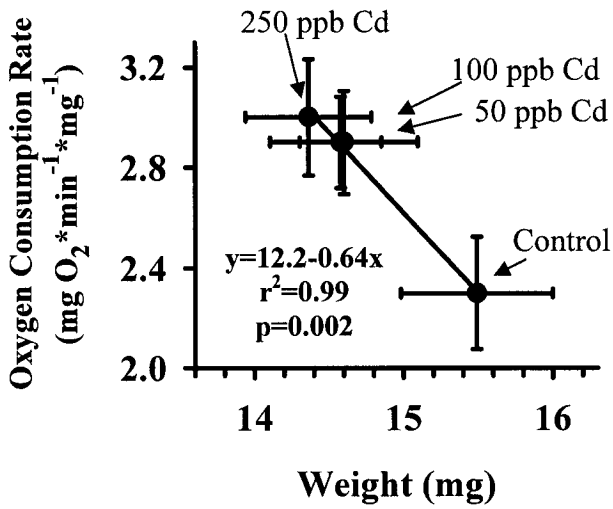


Fig. 13. Mean oxygen consumption rate vs. mean final weight of larval topsmelt exposed to Cd (0-250 ppb) for 14 days. Each data point represents n=8-10/trt.

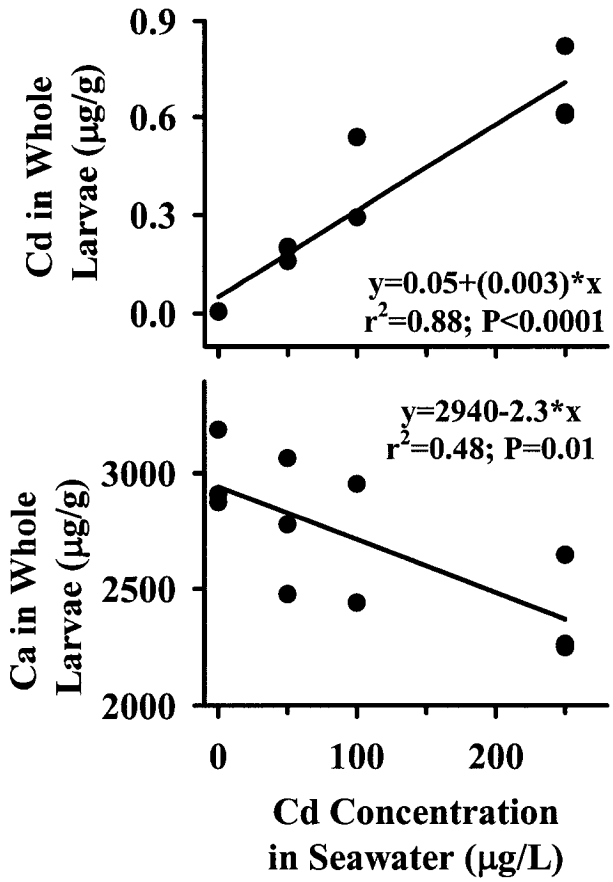


Fig. 14. Cd concentration (top) and Ca concentration (bottom) in whole topsmelt larvae following a 14-day exposure to Cd vs. Cd concentration in seawater.

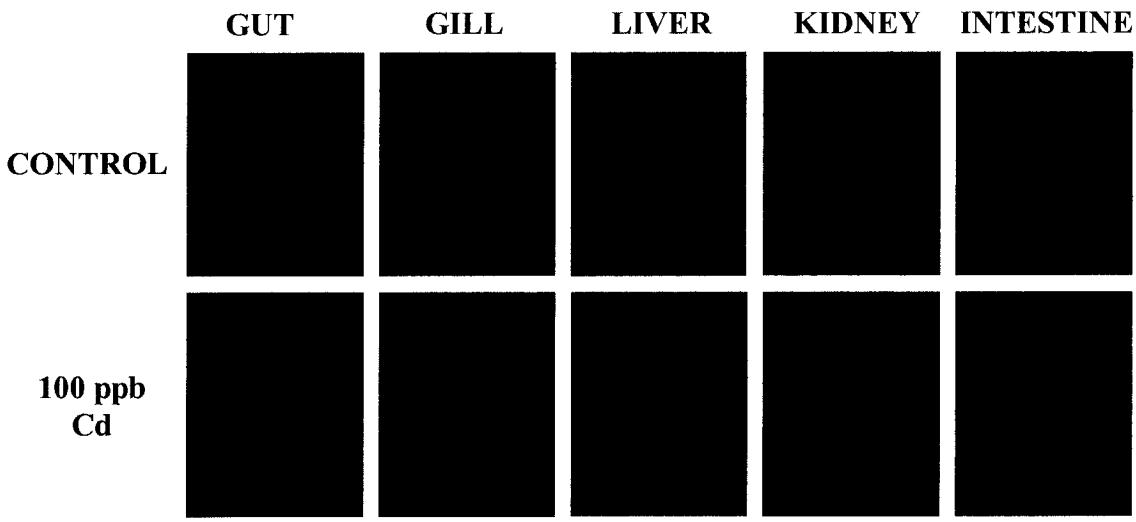


Fig. 15. TUNEL positive cells (+ 1 SE), or those demonstrating DNA fragmentation, in gut (esophagus and stomach), gill, intestine, liver and kidney of larval topsmelt exposed to seawater alone (control) or Cd (100 ppb) for 14 days. TUNEL positive cells had nuclei incorporated with fluorescein-dUTP, whereas all cell nuclei demonstrate staining with Hoechst 33258 (blue).

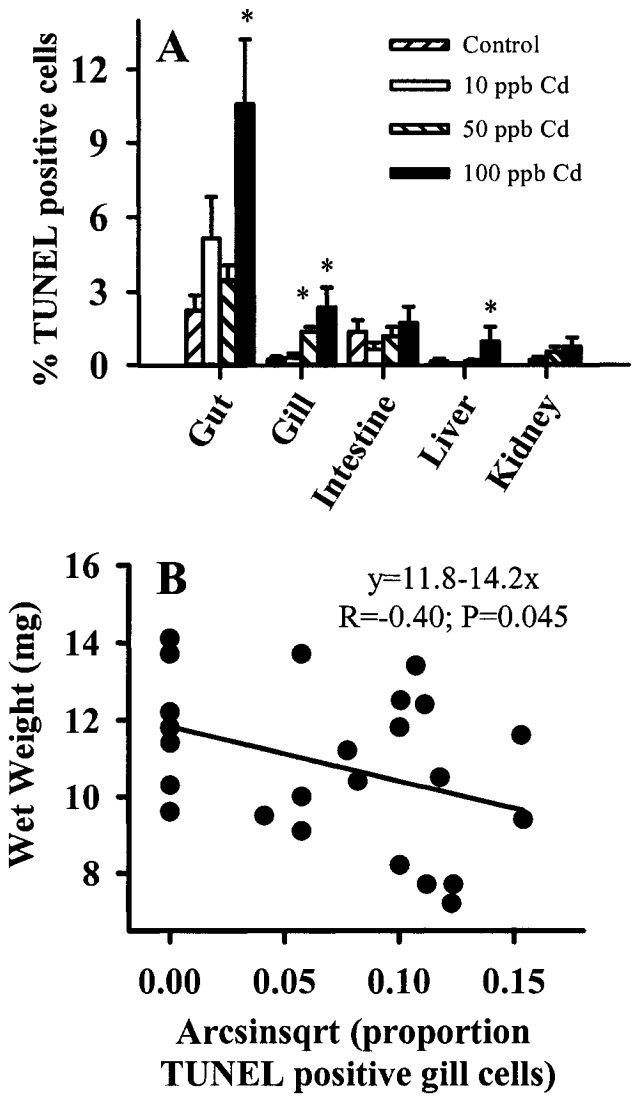


Fig. 16. Percent TUNEL positive cells (+ 1 SE) in gut (esophagus and stomach), gill, intestine, liver and kidney of larval topsmelt exposed to Cd (0 to 100 ppb) for 14 days (A), and the relationship between final wet weight and proportion of TUNEL positive cells in the gill of topsmelt after 14 days of Cd exposure (B). Asterisks indicate significant differences among Cd treatments for each tissue separately at $P<0.05$ (n=5-8/trt).

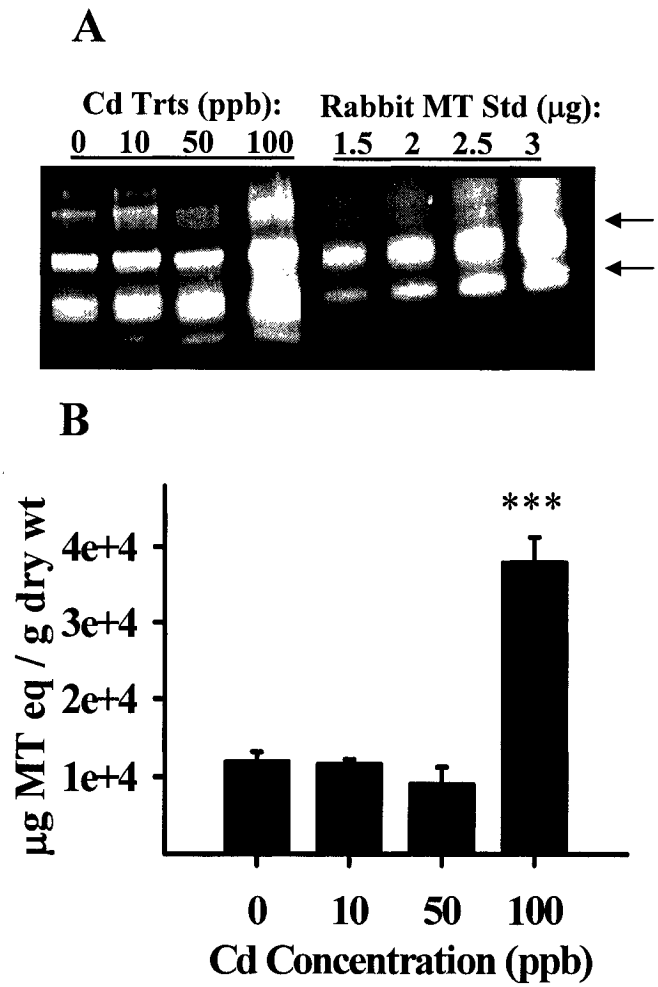


Fig. 17. Levels of metallothionein (MT) - like proteins (A) or MT equivalents (B) in larval topsmelt (n=3 pools of 7-8 fish/trt) exposed to Cd (0 to 100 ppb) for 14 days. One-way ANOVA and SNK multiple comparisons were used to detect differences in mean levels of MT-like equivalents among Cd treatments. Asterisks indicate significant differences at $P<0.05$.

# CHAPTER 3

## Validation of otolith growth rate analysis using cadmium-exposed larval topsmelt (*Atherinops affinis*)

**Abstract**

I applied otolith growth rate analysis to an investigation of cadmium (Cd)-exposed larval topsmelt (*Atherinops affinis*) to determine if growth rate was a more sensitive measure than growth (body weight or length). Topsmelt otoliths, calcareous concretions in the fish inner ear, formed daily increments and otolith growth was proportional to somatic growth. Nine day post-hatch larval topsmelt were exposed to Cd (0 – 100 ppb) in seawater for 14 days, and were fed low or high ration levels in separate experiments. Cadmium impaired topsmelt growth and growth rates; however, the extent of growth reduction was dependent on ration level and growth rates prior to Cd exposure. At low ration levels, fish exposed to Cd (50 and 100 ppb) were smaller in final mean length and weight than controls. At high ration levels, marginal differences in final mean lengths, and no differences in weight were found. However, at both ration levels, otolith and body length growth rates were reduced during Cd (50 and 100 ppb) exposures. Otolith growth rate analysis was more sensitive than growth measurements of Cd-exposed larval topsmelt because it allowed for the detection of small differences in growth rates, even when differences in somatic growth were not observed.

**Introduction**

Growth rate and size are important determinants of survivorship for larval fishes, according to the growth-mortality theory of fish biologists (Houde, 1987; Anderson, 1988). Specifically, a strong relationship exists between increasing size and decreasing mortality during the early life of fishes. Fish larvae with high growth rates may have higher survivorship because of superior developmental fitness, predator avoidance, and

prey foraging, relative to slower growing fish within a cohort. Thus, small changes in growth rate may lead to variable recruitment success, or the number of individuals surviving to a specific stage in their life cycle such as metamorphosis from larvae to juveniles (Houde, 1987). A number of environmental factors such as temperature and food availability may influence growth rates, and ultimately survival of fish larvae (Barkman and Bengtson, 1987; Gleason and Bengtson, 1996). Toxicant exposure also may lead to a reduction in growth rates of fishes and subsequent recruitment processes; however, little is known about how growth rate reductions of toxicant-exposed fishes lead to alterations in fish population size or structure.

Growth is an ecologically significant endpoint that has been used for decades in toxicological investigations with fishes (Woltering, 1984). Biochemical and physiological markers of toxicity, often evaluated in fishes from field-contaminated sites, have greater ecological implications if they are associated with impaired growth, an endpoint indicative of fitness. In toxicity testing, the ability to detect growth reductions at exposure levels below which survival is reduced in short-term experiments may lead to more accurate determinations of the no observable effect concentration (NOEC), and thus provide for improved protection of fish populations (USEPA, 1985). Growth has typically been measured as the final mean weight and final mean length of fishes at the end of an exposure period (USEPA, 1995). The change in weight and length of individual fishes, more sensitive endpoints of growth than final mean size, have been used in studies with post-larval fishes, but rarely with newly hatched larvae as a result of their frailty out of water (Eaton et al., 1978; Rombaugh and Garside, 1982; Goodman et al., 1992; Marr et al., 1996). While individual growth rate measurements are more informative than final

mean weight or length, because they account for changes in growth over time, growth rate is often not evaluated in toxicological studies. Methods are available for measuring growth rates in fish larvae such as the RNA/DNA ratio method, radiocarbon uptake method, and back-calculation from rings on hard structures (Busacker et al., 1990). However, it is unclear whether these methods are applicable to either routine toxicity testing or studies on toxicant-impaired growth rate of larval fishes.

Methods are needed for evaluating subtle changes in growth rates of fishes exposed to toxicants because growth, measured as final mean size or change in size, is often an insensitive endpoint of toxicity. Some studies have only detected a reduction in growth at levels of toxicants that also cause significant mortality (Eaton, 1974; Hollis et al., 1999; Hansen et al., 2002), while others have failed to detect toxicant-impaired growth even at lethal concentrations (Eaton et al., 1978; Brown et al., 2002). Moreover, in a review of 173 fish chronic and early life stage tests, Woltering (Woltering, 1984) found that survival and reproduction were the most sensitive endpoints of toxicity in 30 and 15% of the tests, respectively, in comparison to larval growth, the most sensitive endpoint in only 14% of the tests. Growth rate may be a more sensitive endpoint than final mean size or change in weight or length; however, little is known about how toxicant exposure may lead to changes in growth rates of fishes.

Fish biologists have used otolith analysis since the 1970s to examine daily growth rates and to age fishes (Pannella, 1971). Otoliths, calcareous concretions in the inner ear of fish, are useful endpoints of growth because they contain rings, or increments, that represent a daily endogenous deposition of protein and calcium carbonate (Pannella, 1971). The change in otolith size or increment widths (i.e. on a daily basis) can be used as

a proxy for somatic growth because otolith and fish size are proportional. Thus, fine scale changes in somatic growth, or fish size-at-age, can be back-calculated from otolith increment measurements (Campana, 1990). Otoliths of some Atherinid species such as Atlantic silversides (*Menidia menidia*) have been validated for use in age and growth studies (Barkman, 1978); however, to my knowledge, validation has not been completed for otoliths of topsmelt (*Atherinops affinis*), a model marine species, used in growth and survival tests required by the EPA in effluent toxicity studies (USEPA, 1995). While otoliths have been used to investigate how environmental stressors such as temperature, oceanographic processes, and food availability alter fish growth rates (Barkman and Bengtson, 1987; Moksness et al., 1995; Ralston, 1995), otolith growth rate analysis has not been applied to studies of toxicant effects on growth rates of toxicity test species. The use of the otolith growth rate analysis method for studies in aquatic toxicology may provide for better linkages between toxicity testing and field assessment of biological effects of toxicants at individual and population levels.

In this study, the overarching goal was to determine whether otolith growth rate analysis was more sensitive than standard methods for measuring growth. My first objective was to validate the periodicity of otolith increments in larval topsmelt from 0 to 23 days post-hatch (dph), under laboratory conditions that are typical of toxicity tests with this species. Topsmelt were chosen because they are native to California, and they are a model marine species used in EPA-required toxicity tests (USEPA, 1995). In addition, topsmelt are often found in contaminated California tidal marshes, which they use for nursery habitat, prey foraging, and predator avoidance. My second objective was to apply otolith growth rate analysis to an investigation of cadmium (Cd)-exposed larval

topsmelt (*Atherinops affinis*) to determine if growth rate was a more sensitive measure than growth (body weight or length). Cadmium (Cd) was of interest as a model toxicant in this study because it is well-known to impair the growth of larval fishes (Eaton et al., 1978; Rombaugh and Garside, 1982; Woodworth and Pascoe, 1982; Miliou et al., 1998), and is an environmentally relevant contaminant in California estuaries such as Stege Marsh in San Francisco Bay (Peter Green, personal communication), where pollutant levels, and biochemical and ecological effects are currently under investigation by the Pacific Estuarine Ecosystem Indicator Research consortium (PEEIR). As part of the validation, I also evaluated the sensitivity of otolith growth rate analysis for detecting differences in growth rate of Cd-exposed topsmelt provided with high vs. low ration levels. In the aquatic environment, food availability and toxicant concentration are well-known to vary extensively in time and space. Moreover, alterations in ration level may influence both otolith and fish growth in both field and laboratory investigations (Gleason and Bengtson, 1996), and high ration levels may enable fishes to compensate for toxicant exposure (Hopkins et al., 2002).

## Methods

### *Fish and Water Maintenance*

Larval topsmelt ages 1 to 9 days post-hatch (dph) were purchased from Aquatic Bio Systems (Fort Collins, CO) and acclimated under experimental conditions for 24 hr. Water quality (1-μm-filtered seawater at 33 ppth, 20°C, pH = 7.8-8.3, dissolved oxygen > 7 mg/L) and photoperiod (16:8, L:D) were maintained according to topsmelt toxicity

testing protocols (USEPA, 1995) and fish were fed *Artemia* nauplii *ad libitum* prior to experimentation.

*Otolith Increment Periodicity*

Otolith increment periodicity was investigated by (1) determining the sagittal otolith size and number of increments in otoliths of cultured topsmelt at age zero, (2) examining increment periodicity in otoliths of larval topsmelt marked with alizarin red S, and (3) determining if there was a constant proportionality between otolith size and topsmelt size from ages 8 to 23 dph, necessary for back-calculations of size-at-age. To assess the size and number of increments in sagittal otoliths of topsmelt at hatching, I obtained 0 dph larval topsmelt (n=10) fixed in ethanol, removed the sagittal otoliths, and mounted otoliths in Crystalbond 509 (Aremco Products, Inc., Valley Cottage, NY) on slides. A Zeiss compound microscope (Carl Zeiss MicroImaging, Thornwood, NY) with a Spot camera (Diagnostic Instruments, Inc., Sterling Heights, MI) was used to capture images of otoliths at 400X magnification. Image Pro® software was used to measure otolith radii and surface areas and count increments within each otolith. Linear regression was used to examine the relationship between length and otolith radii at age zero (Sokal and Rohlf, 1981).

To determine increment periodicity in topsmelt otoliths, 9 dph larval topsmelt (n=10) were exposed to a NaOH-buffered solution of 100 mg/L alizarin red S (ARS, Sigma-Aldrich, St. Louis, MO) in 500 mL of seawater (33 ppth) for 24 hr (Lagardère et al., 2000). During and subsequent to ARS exposures, fish were fed *Artemia* nauplii *ad libitum*. At 16 dph, fish were placed again in 100 mg/L ARS for 24 hr. After the second

ARS exposure, fish were sacrificed on ice and rinsed in deionized water. Both sagittal otoliths were extracted from each fish under a stereoscopic dissecting scope and mounted in Crystalbond 509 on slides until further analyses. Images of otoliths were captured under visible and fluorescent (ex/em = 359/461 nm) light at 100X and 400X magnifications using an Olympus BX50WI fixed stage upright microscope (Olympus America, Melville, NY) with a Cool Snap HQ Camera (Photometrics, Tucson, AZ). Daily increments between ARS exposures were validated for each fish using Image Pro® software. Light and fluorescent images of each otolith were compared and otolith increments were counted along the ventral radius of each otolith between ARS exposures.

To examine the otolith-length relationship for topsmelt, 8 dph larval topsmelt (n=72) were randomly placed in 200 mL of seawater in 500 mL polypropylene beakers. Water changes were performed every other day and water quality was maintained as described above. During the experiment, fish were fed 80 *Artemia* nauplii from 8 to 17 dph and 160 *Artemia* nauplii from 17 to 23 dph. Fish (n=12) were sacrificed on ice at 8, 11, 14, 17, 20 and 23 dph, weighed and measured. Sagittal otoliths were removed and mounted and light microscopy was used to capture images of otoliths at 160X magnification as described above. Image Pro® software was used to verify the number of otolith increments at each sampling time (8 to 23 dph), and the increment width per day on the ventral side of the otolith from hatch to time of sampling (0 to 23 dph). The final size of the ventral radii and surface area of each otolith was measured. Linear and non-linear regressions were performed to examine the relationships among length, weight, otolith radii, and otolith surface areas (Sokal and Rohlf, 1981).

*Validation of Otolith Growth Rate Analysis Using Cd-Exposed Larval Topsmelt*

*Experiment 1.* Topsmelt larvae (n=8/trt) were aqueously exposed in a static renewal system (500 mL polypropylene beakers) to seawater alone (200 mL) or to 10-100 μg/L (ppb) Cd in seawater for 14 days. Each test solution was prepared by adding a $CdCl_2$ stock solution to 1-μm-filtered natural seawater from the Bodega Marine Laboratory in Bodega Bay, CA, where the experiments were conducted. Sublethal levels of aqueous Cd exposure in seawater were based on the results of a 7-day mortality study in our laboratory ($LC_{50}$ = 470 ppb). Water changes were performed every other day and water was maintained as described above. Fish were fed 160 *Artemia* nauplii per day, or low ration levels.

*Experiment 2.* Topsmelt larvae (n=24/trt) were aqueously exposed to Cd as described in experiment one except that fish were fed 190 *Artemia* nauplii per day, or high ration levels. A larger sample size (n=24/trt) was used in this experiment to improve the power to detect differences in final size and growth rates among treatments.

*Otolith Analysis*

At 23 dph, the wet weight and length of larval topsmelt were determined, and sagittal otoliths were removed and mounted as described above. Using Image Pro® Plus software (Media Cybernetics, Inc., Silver Spring, MD), the final otolith radius and surface area size of each otolith were measured. The daily otolith radius sizes were measured from 1 dph to 23 dph on the ventral side of the otolith and otolith surface areas were measured at 1, 9, and 23 dph. Daily otolith radii and surface area growth rates were determined before and during Cd exposure by calculating the average increment width

per day and surface area growth per day from before (1 to 9 dph) to during (9 to 23 dph) Cd exposure.

*Calculation of Topsmelt Length From Ages 1 to 22 dph*

Fish length from 1 to 22 dph was estimated from otolith ventral radii using the Biological Intercept Model (Campana, 1990). The equation used was: fish length $_{@\ age\ i}$ = length $_{@\ 23\ dph}$ + (((otolith radius $_{@\ age\ i}$ – otolith radius $_{@\ 23\ dph}$)*(length $_{@\ 23\ dph}$ – approximate length $_{@\ 0\ dph}$))/(otolith radius $_{@\ 23\ dph}$ – otolith radius $_{@\ 0\ dph}$)), where, i = 1 to 22 dph. The otolith radius at 0 dph (Biological Intercept) was determined for each individual fish from their otoliths and the length of each individual fish at 0 dph was estimated from the otolith-length regression determined in the validation portion of this study (Fig. 18). Daily length growth rates both before and during Cd exposures were calculated as follows: Before = (9 dph – 1 dph length)/8 days; During = (23 dph – 9 dph length)/14 days.

*Estimation of Weight of Topsmelt From Ages 1 to 22 dph*

A non-linear regression was performed to examine the relationship between weight and length using data from all fish in the growth standard curve experiment and fish from the control treatments in the Cd exposure experiments. This equation (weight = $(0.0011)*(\text{length})^{3.52}$; $r^2 = 0.98$; $P<0.001$) was used to estimate the weight of fish at 1 dph and 9 dph. Weight growth rates both before and during the Cd exposures were calculated as follows: Before = (9 dph – 1 dph weight)/8 days; During = (23 dph – 9 dph weight)/14 days.

*Statistics for Cd-Exposure Experiments*

Data was log-transformed when necessary and one-way fixed-factor ANOVAs and Student-Newman-Keuls (SNK) multiple comparisons were used to detect differences in the final mean length, weight, otolith radii, and otolith surface areas of larval topsmelt within different Cd treatments for each experiment and endpoint, separately (Sokal and Rohlf, 1981). Two-sample t-tests were used to determine if there were differences in length, weight, otolith radii, and otolith surface areas of fish fed high rations (190 *Artemia* nauplii per day) vs. those fed low ration levels (160 *Artemia* nauplii per day) for each endpoint separately. Analyses of co-variance (ANCOVAs) and SNK multiple comparisons were used to detect differences in otolith radii and length growth rates, among Cd treatments, using before Cd exposure growth rate as the co-variate in each analysis. To examine differences in weight and otolith surface area growth rates among treatments, I calculated the difference in growth rate (during – before Cd exposure growth rates). One-way ANOVAs and SNK multiple comparisons were used to detect differences in the magnitude of growth rate change among treatments for each experiment and endpoint, separately. Here, I chose ANOVA and ANCOVA as statistics because these tests were more powerful than regression analysis for comparing the sensitivity of otolith growth rate analysis to standard growth measurements among fish from each Cd treatment level. Two-sample t-tests were used to measure differences in otolith radii and length growth rates of fish fed high ration levels relative to those fed low ration levels. Two-sample t-tests were also used to examine differences in otolith surface

area and weight growth rate changes from before to during Cd exposure of fish fed high ration levels vs. those fed low ration levels.

*Cd Chemistry*

In experiments one and two, separately, water samples were randomly taken from two beakers in each Cd concentration level both before and after one water change, for a total of n=4/trt. Samples were stored in the dark at 4°C until just prior to analysis. Samples were brought to room temperature, gently stirred to return condensed vapor to the solution in the bottom of the tube. Samples (250 µl) were diluted 20-fold in 1% nitric acid (Trace Metal grade, Fisher Scientific, Pittsburgh, PA) in a new polypropylene centrifuge tube. A blank tube was measured as a control, confirming negligible contamination. Quantification was by direct aspiration in an ICP-MS (model 7500i, Agilent, Palo Alto, CA) and compared to external standards (traceable to NIST) covering a range from 0.1 µg/L to 20 µg/L. In the fashion prescribed by EPA method 6020, the instrument detection limit (triple the standard deviation of blanks) was 0.006 µg/L corresponding to a method detection limit of 0.12 µg/L. Both Cd isotopes 111 and 114 were measured, and elements Mo and Sn were monitored to ensure negligible interference. The instrument was operated with a robust plasma characterized by 0.4% CeO/Ce.

## Results

*Otolith Increment Periodicity*

I detected 9±0.5 increments (n=9) in sagittal otoliths of age zero larval topsmelt, and determined that otolith size was positively correlated with length of age zero topsmelt (Table 4, Fig. 18). Larval topsmelt exposed to alizarin red S (ARS) demonstrated rings of ARS fluorescence under UV epi-illumination from 9 to 10 and 16 to 17 dph that corresponded to otolith increments under visible light (Fig. 19). Otolith increment formation occurred on a daily basis between the two ARS exposures for all (n=10) ARS-exposed topsmelt (Fig. 19C).

Sagittal otoliths from larvae ages 8 to 23 dph demonstrated a check on the otolith that was indicative of hatching and approximately 9 increments were detected before this check (Figs. 18, 19A). Using this hatching check to determine size at age zero, I measured daily otolith increments on the ventral side of the sagittal otoliths in topsmelt larvae ages 8 to 23 dph. Some variability in daily increments (± 1 increment) was found when otolith radii were measured from the core to the rostrum, anti-rostrum or dorsal margins of the otoliths. At high magnification (> 400X), sub-daily increments also were visible with approximately one sub-daily increment between each daily increment (Fig. 19C).

Significant exponential relationships were found between weight vs. length and otolith radii (Table 4, Fig. 20A). A linear fit best described the relationship between weight and otolith surface areas (Table 4). Otolith radii were positively correlated to length of larval topsmelt from 8 to 23 dph (Table 4, Fig. 20B); However, an exponential curve best fit the relationship between otolith surface area and length (Table 1, Fig. 20C).

*Validation of Otolith Growth Rate Analysis Using Cd-Exposed Larval Topsmelt*

Fourteen-day aqueous Cd exposure led to diminished final mean length, weight, and otolith size of larval topsmelt relative to that of controls (Figs. 21, 22). The effects of Cd were dependent in part on the Cd concentration, and the ration level, or the number of *Artemia* nauplii fed to topsmelt larvae. Length, weight, otolith radii, and otolith surface areas of fish fed high rations (190 *Artemia* nauplii per day) were significantly larger than those fed low ration levels (160 *Artemia* nauplii per day; Two-sample t-tests, $P<0.001$). At low ration levels, length and weight of control fish were significantly greater than length and weight of fish exposed to 50 and 100 ppb Cd (ANOVAs, $P<0.05$). Otolith radii of control fish were significantly larger than those of fish exposed to 50 and 100 ppb Cd (ANOVA, $P<0.05$). However, significant differences in otolith surface areas were not detected among treatment groups in this experiment (ANOVA, $P=0.125$).

At high ration levels, final mean otolith radii of control fish were significantly larger than otolith radii of fish exposed to 50 ppb Cd ($P=0.01$) and marginally larger than those of fish exposed to 100 ppb Cd ($P=0.09$). Similarly, final mean length of control fish was significantly greater than length of fish exposed to 50 ppb Cd ($P=0.01$) and marginally greater than those of fish exposed to 100 ppb Cd ($P=0.09$). Differences in final mean weight and otolith surface areas, separately, were not detected among fish within different Cd treatments in this experiment (ANOVAs, $P>0.20$).

Otolith radii and length growth rates were significantly faster in fish fed high ration levels relative to those fed low ration levels (Two-sample t-tests, $P<0.001$). Regardless of ration level, fish exposed to Cd demonstrated diminished length and otolith

radii growth rates, relative to fish in control treatments (Fig. 23). At both low and high ration levels, separately, otolith radii growth rates of fish in 50 and 100 ppb Cd treatments were significantly reduced during Cd exposure relative to those in control or 10 ppb Cd treatments (ANCOVAs, $P<0.05$). Similarly, at both ration levels, length growth rates of fish in 50 and 100 ppb Cd treatments were significantly reduced during Cd exposure relative to those in control or 10 ppb Cd treatments (ANCOVAs, $P<0.01$).

Otolith surface area and weight growth rate increases from before to during Cd exposure were significantly larger in fish fed high rations relative to those fed low rations (Two-sample t-tests, $P<0.01$). In addition, Cd exposure had an effect on the magnitude of otolith surface area and weight growth rate increases from before to during Cd exposure (Fig. 24). At both low and high ration levels, otolith surface area growth rate increases from before to during Cd exposure were significantly smaller in fish exposed to 50 and 100 ppb Cd, relative to growth rate increases of fish from control or 10 ppb Cd treatments (ANOVAs, $P<0.05$). Similarly, regardless of ration level, weight growth rate increases from before to during Cd exposure were significantly smaller in fish exposed to 50 and 100 ppb Cd, relative to growth rate increases of fish from control or 10 ppb Cd treatments (ANOVAs, $P<0.05$).

*Cd Chemistry*

In experiment one, mean Cd concentrations ($\pm$ 1 SD) that corresponded to 0, 10, 50 and 100 ppb nominal Cd concentrations randomly sampled from four beakers per treatment were as follows: <0.1, $10.5 \pm 2.7$, $52.8 \pm 3.2$, and $118 \pm 4.5$ ppb. In experiment two, mean Cd concentrations ($\pm$ 1 SD) that corresponded to 0, 10, 50 and 100 ppb

nominal Cd concentrations sampled from four beakers per treatment were as follows: $0.06 \pm 0.02$, $9.1 \pm 0.3$, $44.8 \pm 1.4$, and $83.8 \pm 6.0$ ppb.

## Discussion

### *Otolith Increment Periodicity*

I found that (i) otoliths were present at hatch and had $9 \pm 0.5$ increments, (ii) increment formation occurred on a daily basis in otoliths of larval topsmelt marked with alizarin red S, and (iii) there was constant proportionality between otolith size and topsmelt larvae size from ages 8 to 23 dph. The presence of topsmelt otoliths containing numerous increments at hatch suggests that the formation of the first otolith increment occurred before hatch in this species; however, otoliths were not directly examined during embryonic development of topsmelt in this study. In inland silversides (*Menidia beryllina*), a closely-related atherinid species, the formation of the first otolith increment was shown to occur approximately three days after fertilization (Gleason and Bengtson, 1996). The formation of the first otolith increment varies widely among species; increments were detected on the day of hatching in walleye pollock (*Theragra chalcogramma*) (Nishimura and Yamada, 1984), and several days after hatching in delta smelt (*Hypomesus transpacificus*) (Jim Hobbs, personal communication). In my investigation, it was necessary to determine the starting size and increment number of topsmelt otoliths at hatch such that daily increments could be accurately counted from the initial starting size to the final size of each otolith at the end of an experiment.

The otolith increment periodicity, or daily ring accumulation determined in topsmelt in this study, has been measured in many distantly- and two closely-related

species (Barkman, 1978; Gleason and Bengtson, 1996; Campana and Thorrold, 2001). Daily otolith increments of laboratory-reared Atlantic silversides were detected in fish ages 15 to 68 dph and were related to age (Barkman, 1978). The presence of daily otolith increments in inland silversides, was determined in field-collected fish by back-calculations to hatch date, based on the gonadosomatic index peak (Gleason and Bengtson, 1996). In both *Menidia* species, as well as most species whose otoliths have been validated to date, variation in increment periodicity has been reported. Here, I had little variation in daily periodicity possibly because daily rings were measured in laboratory-reared topsmelt over a short time period, and increments were measured from the core to the most ventral margin of each topsmelt otolith. When otolith increment measurements were taken from core to rostrum, to anti-rostrum or to dorsal margins of the otolith, greater variation in increment periodicity was found (data not shown).

Larval topsmelt otoliths grew isometrically relative to length from 8 to 23 dph, which is similar to *Menidia* species. In field-collected Atlantic silversides ranging from approximately 15 to 80 mm, otolith diameter was significantly linearly correlated with length (Barkman and Bengtson, 1987). Similarly, otolith length was highly correlated with standard length of inland silversides ranging from 5 to 15 mm, regardless of ration level (Gleason and Bengtson, 1996). The determination of a constant proportionality between otolith and fish growth in my study was important because it is one assumption of the Biological Intercept Model, used to back-calculate length at different ages of fishes based on otolith increments (Campana, 1990). In contrast to length-otolith relationships, the relationship between weight vs. length of larval topsmelt was best described by an exponential equation, indicating that weight growth was greater than length growth for

topsmelt from 8 to 23 dph. The exponential relationship between length and weight is typical of early life stages of fishes examined over short time periods (Ricker, 1979).

*Validation of Otolith Growth Rate Analysis Using Cd-Exposed Larval Topsmelt*

In this study, I found that otolith growth rate analysis was a more sensitive method than standard growth measurements of Cd-exposed larval topsmelt, because it allowed for the detection of small differences in growth rates, even when differences in somatic growth were not found. At low ration levels, fish exposed to 50 and 100 ppb Cd were significantly smaller in final mean length and weight than controls, whereas at high ration levels, there were no significant differences in final mean weight and marginal differences in final mean length of larval topsmelt among treatments. I determined that at both ration levels, otolith and length growth rates of fish were significantly slower during Cd (50 and 100 ppb) exposure relative to controls, when I accounted for growth rates prior to Cd exposure. I also found significant differences in the magnitude of the weight and otolith surface area growth rate increases in fish from before to during Cd (50 and 100 ppb ) exposure in fish relative to controls.

To my knowledge, this study was the first to apply otolith growth rate analysis to a toxicological investigation with the model marine species, topsmelt, used in growth and survival tests required by EPA for examining effluent toxicity (USEPA, 1995). Otolith analysis provided me with the power to detect differences in growth rates of Cd-exposed larval topsmelt relative to controls, particularly at high ration levels, when differences in final mean size among treatments were otherwise not significant. Two studies to date, have examined otolith growth in fishes exposed to toxicants in the laboratory. In early

life stage tests, Humphrey et al. (Humphrey et al., 2003) found differences in otolith perimeter and length of Eastern rainbowfish (*Melanotaenia splendida splendida*) after 14 days of chlorpyrifos exposure, but only at concentrations that also caused significant mortality. Moreover, the effects of sublethal levels of chlorpyrifos on otolith and fish growth rates, more sensitive endpoints than final mean size, were not determined in Eastern rainbowfish (Humphrey et al., 2003). Significant differences in post-emergence otolith growth were found in juvenile pink salmon (*Oncorhynchus gorbuscha*) exposed to dietary crude oil for 6 weeks (Mortensen and Carls, 1995). Here, the implications of impaired otolith growth on the fish physiology and condition were unclear, because otolith growth did not correspond to somatic growth of pink salmon, and otolith increments were not daily. In addition to measuring otolith and fish final size and evaluating otolith growth during exposure to toxicants, I used otolith analysis to evaluate growth rates of topsmelt before and during Cd exposure. Moreover, because otolith growth corresponded well with somatic growth of larval topsmelt, I was able to back-calculate length and weight growth rates based on daily otolith increments.

These results demonstrate that otolith and somatic growth rates, which change during each life stage of fishes (Ricker, 1979), are more sensitive endpoints of toxicity than measurements of final mean size or changes in weight or length (data not shown). Specifically, while significant differences in growth rate of Cd-exposed fishes were found regardless of ration level, differences in final mean size and change in size were marginally or not significant. Because many studies have failed to detect growth impairment in toxicant-exposed fishes at levels below which survival is reduced (Eaton, 1974; Eaton et al., 1978; Woltering, 1984; Hollis et al., 1999; Brown et al., 2002;

Hansen et al., 2002), it has been argued that growth measurements should be eliminated from toxicity testing protocols. As an alternative, I suggest that somatic growth rate estimates based on otolith growth rate analyses, such as those performed in this study, could replace the standard measures of final mean size and thus provide a more informative and sensitive endpoint of sublethal toxicity. While otolith growth rate analyses are new to the field of aquatic toxicology, otoliths have long been studied by fish biologists to evaluate how environmental variables affect growth rate and survival of larval fishes. Growth rates of larval rockfish, measured by daily otolith increment widths, were associated with fluctuating sea surface temperature, sea-level anomaly, and upwelling and downwelling episodes (Ralston, 1995). Similarly, based on larval growth rate fluctuations of damselfish (*Stegastes partitus*), Wilson and Meekan (Wilson and Meekan, 2002) found that faster growing larvae survived predation and other environmental variables better than slower growing larvae and thus helped to replenish their benthic populations. It is likely that otolith growth rates reflect stress due to toxicant exposure because they are good indicators of environmental stressors.

In my study, fish fed low ration levels demonstrated slower otolith and somatic growth rates than fish fed high ration levels, regardless of Cd concentration. These results provide further evidence that otolith growth rates are a reflection of somatic growth rates and that otolith growth is a good indicator of the nutritional condition of larval topsmelt. While ration level was manipulated in separate experiments in my study, fish in both experiments were reared at the same culture facility and held under analogous laboratory conditions before and during experimentation. The effects of ration level on the size of the otolith and fish were also demonstrated in inland silversides from 0 to 24 dph

(Gleason and Bengtson, 1996), where faster otolith and fish growth rates were found in fish fed higher ration levels. Ration level also affected otolith and fish growth of North sea herring (*Clupea harengus*) larvae held in mesocosms for 60 days (Moksness et al., 1995). Here, increases in prey density, and presumed feeding, were reflected by a higher otolith growth rate, but were delayed 7 days relative to body growth rate.

The substantially lower growth rate impairment in fish provided with high rations relative to those given low rations indicates that fish provided with high rations were able to compensate for exposure to Cd. Previous studies provide evidence that high ration levels may lead to reduced toxicity. In coal-ash-exposed lake chubsuckers *(Erimyzon sucetta*) fed high ration levels, no significant reductions in growth or health were found relative to controls; however, as ration level was reduced, growth, survival, and health were diminished to a greater extent in ash-treated chubsuckers relative to controls (Hopkins et al., 2002). Similarly, more copper accumulated in the liver of starved compared to fed roach (*Rutilus rutilus*) and liver cell pathology was altered to a greater extent in starved fish exposed to copper (Segner, 1987). In these studies, fishes provided with higher ration levels were able to compensate for toxicant exposure possibly because they had a higher scope-for growth (SFG), or the energy available for growth of an organism that is in excess of energy required for maintenance purposes (Widdows, 1985). While SFG has been studied extensively in toxicant-exposed marine invertebrates, little is known about how SFG is altered in toxicant-exposed fishes.

Regardless of ration level, diminished growth rates of topsmelt exposed to Cd (50 and 100 ppb) may have been the result of reductions in energy-yielding processes or increases in energy demand in fishes. Rainbow trout ate less during chronic exposure to

sublethal concentrations of Cd, indicating that growth reductions may be the result of reduced feeding or energy intake (McGeer et al., 2000). Cadmium exposure also may lead to an increased energy expenditure. Diminished growth in rainbow trout chronically exposed to Cd was associated with liver glycogen depletion and increased glycolytic enzyme activities, indicating that there was a higher energy demand in the liver of Cd-exposed fish (Ricard et al., 1998). Alternatively, decreased growth may be the result of an increased use of energy for detoxification purposes such as metallothionein synthesis or increased antioxidant enzyme activity (Olsson et al., 1989).

Reductions in growth rates of Cd-exposed topsmelt suggest that Cd may have altered the energy budget of topsmelt; less energy was allocated for growth because more energy was needed for maintenance purposes such as detoxification or elimination of Cd (Kooijman, 2000; Nisbet et al., 2000). Previous studies have used mathematical models to demonstrate that contaminant-induced alterations in the energy fluxes within individuals may affect the persistence and stability of populations (Nisbet et al., 1997). Thus, growth impairment of larval topsmelt could have important consequences for population dynamics. In a companion paper, dynamic energy budget models (Kooijman, 2000) will be used to determine how Cd changes the rates at which topsmelt acquire and utilize energy for maintenance and growth.

Otolith growth rate analysis may be useful in toxicity testing with topsmelt or other toxicological studies because it allows for the detection of small differences in growth rates between exposed and unexposed fishes. Specific equipment is needed for successful otolith growth rate analyses of larval fishes including mounting media and slides, as well as a visible light microscope, camera, and software for otolith analysis.

Training is also necessary such that otolith increments may be analyzed accurately and are comparable among laboratories (Campana and Thorrold, 2001). Upon the completion of training and the purchase of equipment, the analysis of larval otoliths is cost-effective because larval fish have very few otolith increments to count and measure. In addition, the knowledge of the age of the larval topsmelt allows for further verification of the numbers of otolith increments, and improved accuracy. By accounting for the individual variability in growth rates before toxicant exposure, differences in otolith and somatic growth and growth rates during toxicant exposure may be more easily determined. Thus, otolith analysis may help to improve the current toxicity test method with topsmelt, which fails to account for the highly variable size and growth rates of larval fish prior to toxicant exposure (Ricker, 1979), and likely is associated with higher variability in growth and inadequate power to detect differences in growth among treatments. Nevertheless, additional studies are needed to investigate the use of otoliths to evaluate the growth rates of toxicant-exposed fish over 7 day durations, and to determine whether otolith analysis is useful in examining the effects of additional toxicants and toxic effluents on growth rates of larval topsmelt.

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Table 4. Results of linear and non-linear ($y=a*x^b$) regression analyses among length, weight, otolith radii, and otolith surface areas of larval topsmelt at 0 dph or from 8 to 23 dph.

| Regression | Coefficient | Std. Error | df | F | P | $r^2$ |
|---|---|---|---|---|---|---|
| Otolith Radii vs. Length: 0 dph | 5.32 | 0.99 | 1 | 29.0 | 0.002 | 0.80 |
| Weight vs. Length: 8 to 23 dph | 3.34<br>0.0017 | 0.12<br>0.0005 | 1 | 876 | <0.001 | 0.93 |
| Otolith Radii vs. Length: 8 to 23 dph | 8.9 | 0.55 | 1 | 265 | <0.001 | 0.81 |
| Otolith Surface Area vs. Length: 8 to 23 dph | 2.34<br>101 | 0.10<br>25.7 | 1 | 595 | <0.001 | 0.90 |
| Otolith Radii vs. Weight: 8 to 23 dph | 0.33<br>54.7 | 0.02<br>1.75 | 1 | 381 | <0.001 | 0.86 |
| Otolith Surface Area vs. Weight: 8 to 23 | 3733 | 136 | 1 | 762 | <0.001 | 0.92 |

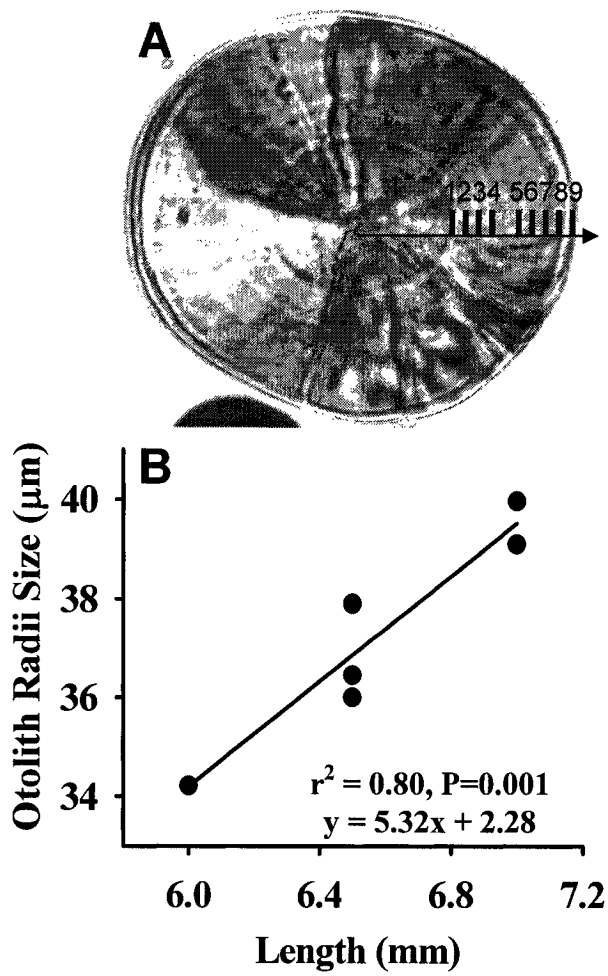


Figure 18. The number of rings on sagittal otoliths (A) and the linear relationship between otolith size and length of larval topsmelt on the day of hatch (B). I detected 9±0.5 rings in otoliths of newly hatched larval topsmelt (n=9).

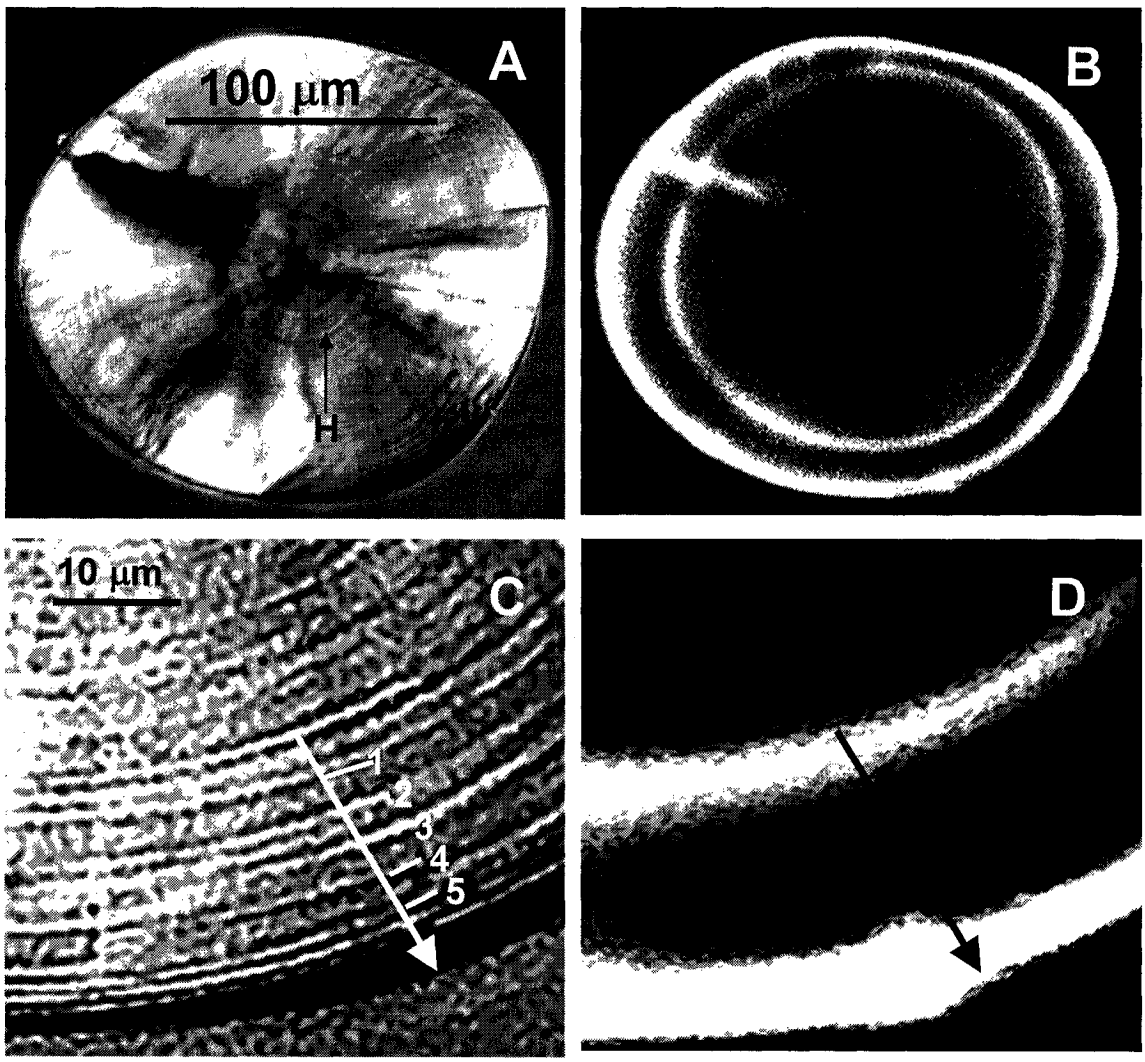


Figure 19. Light (A,C) and fluorescent (B,D) images of sagittal otoliths from larval topsmelt exposed to alizarin red S (ARS) for 24 hr at 9 to 10 days post-hatch (dph) and at 16-17 dph. Images C and D are insets of A and B, respectively. H indicates the hatch mark in the sagittal otolith in image A. The arrow in images C and D is drawn across a transect of otolith rings between the ARS exposures and the numbers indicate the daily rings acquired between ARS exposures.

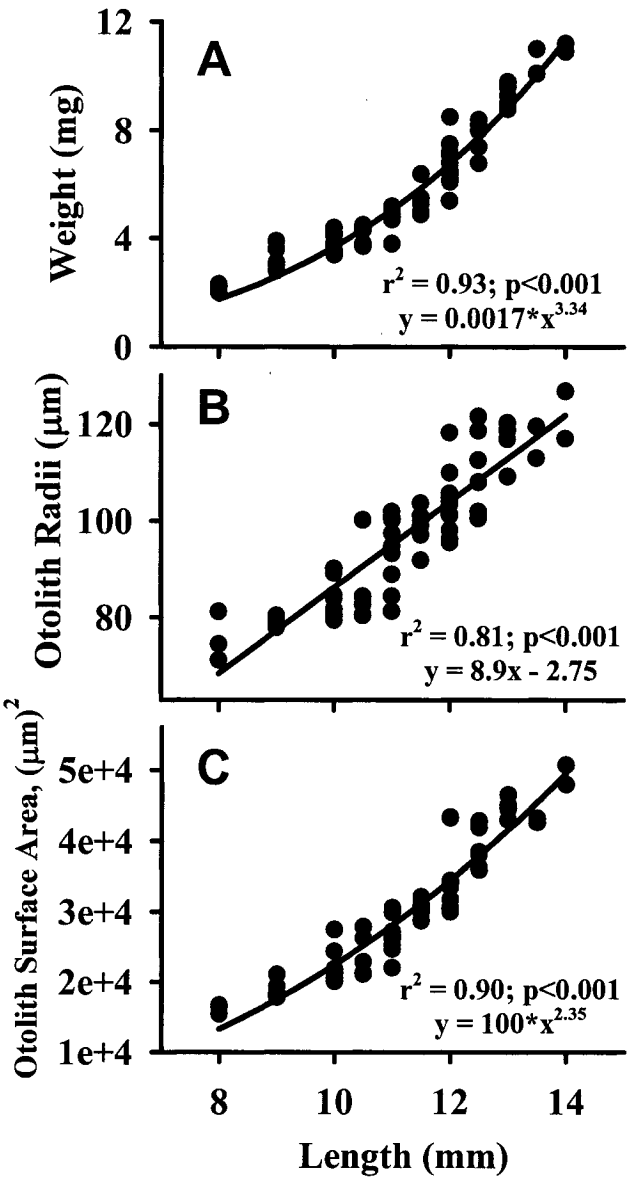


Figure 20. Mean length (± 1 SE) vs. mean weight (± 1 SE) (A), mean otolith radii (± 1 SE) (B), and mean otolith surface area (± 1 SE) (C) of larval topsmelt from ages 8 to 23 dph. Larval topsmelt (n=12/trt) were maintained in individual beakers and sampled every three days to examine the relationships among somatic and otolith growth.

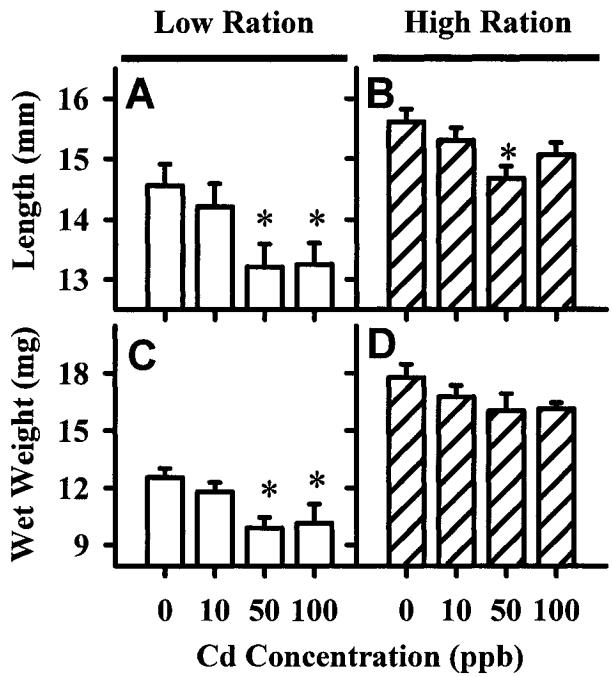


Figure 21. Final mean length (± 1 SE) (A,B) and final mean weight (± 1 SE) (C,D) of 23 dph larval topsmelt exposed to Cd (0-100 ppb) for 14 days. In Experiment 1, fish (n=7-8/trt) fish were fed low ration levels, or 160 *Artemia* nauplii per day, whereas in experiment 2, fish (n=22-24/trt) were fed high ration levels, or 190 *Artemia* nauplii per day. Asterisks indicate significant differences from controls at $P<0.05$.

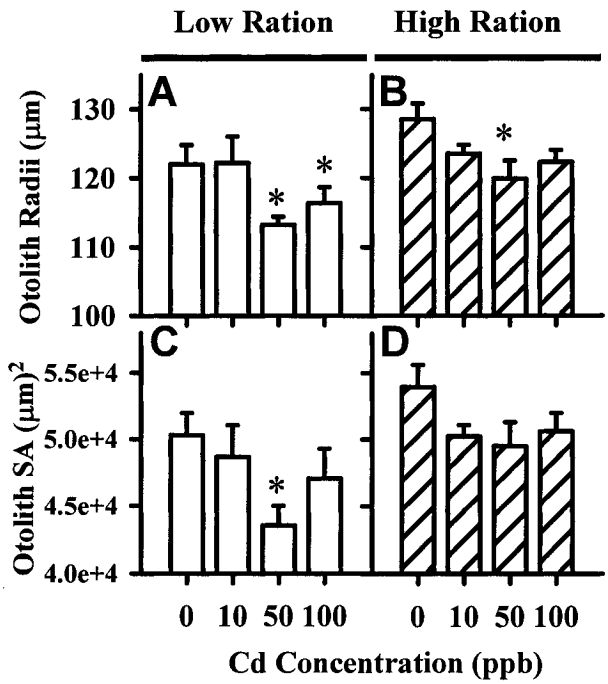


Figure 22. Final mean size of otolith radii (± 1 SE) (A,B) and otolith surface areas (± 1 SE) (C,D) of 23 dph larval topsmelt exposed to Cd (0-100 ppb) for 14 days. Asterisks indicate significant differences from controls at $P<0.05$.

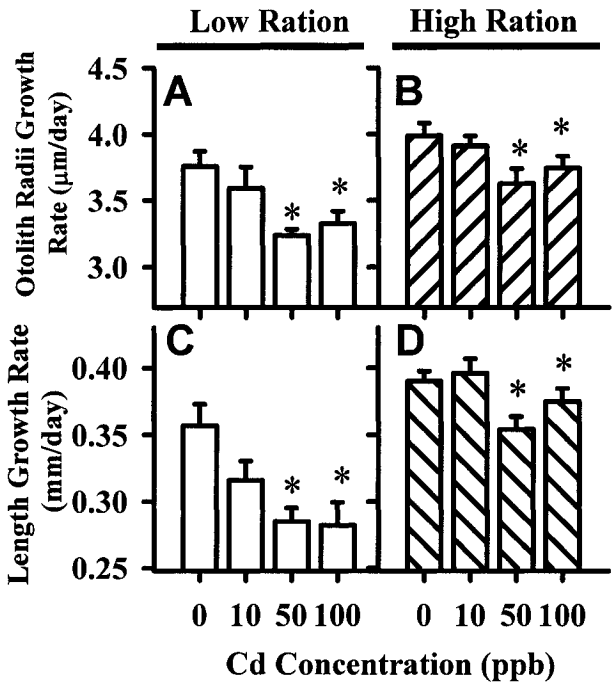


Figure 23. Mean otolith radii (± 1 SE) (A,B) and mean length (± 1 SE) (C,D) growth rates of 23 dph larval topsmelt exposed to Cd (0-100 ppb) for 14 days. Length at ages 1 to 22 dph was back-calculated using the biological intercept procedure (Campana, 1990). Asterisks indicate significant differences from controls at $P<0.05$.

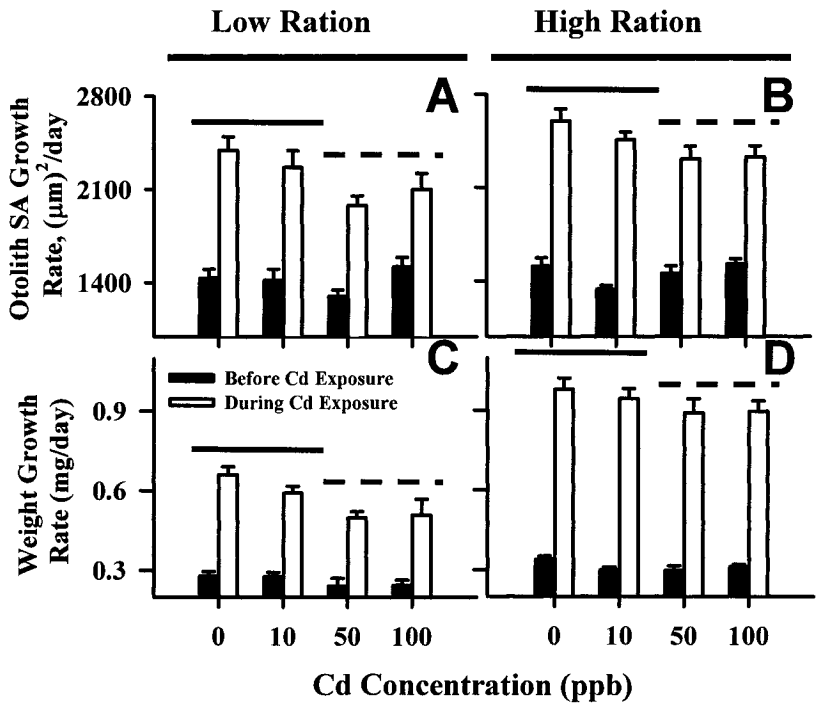


Figure 24. Mean otolith surface area (± 1 SE) (A,B) and mean weight (± 1 SE) (C,D) growth rates of 23 dph larval topsmelt exposed to Cd (0-100 ppb) for 14 days. Weight at ages 1 to 22 dph was estimated based on length to weight relationships of larval topsmelt (weight = $(0.0011)*(\text{length})^{3.52}$; $r^2 = 0.98$; $P<0.001$). Dashed lines indicate significant differences in growth rate increases from before to during Cd exposure among treatments at $P<0.05$ relative to growth rate increases indicated by the solid lines, for each endpoint separately.

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