THE EFFECTS OF CADMIUM ON THE OLFACTORY SYSTEM OF LARVAL ZEBRAFISH

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By

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Keywords: zebrafish larvae, olfactory, cadmium, calcium, transgenic, hsp70

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Abstract

Cadmium (Cd) is a toxic metal known to accumulate in and have adverse effects on the olfactory systems of fish. The objective of this project was to investigate the effect of cadmium on zebrafish larvae, specifically the effects on the olfactory system at cellular and functional levels. Zebrafish larvae (72 hours post fertilization) were exposed to sublethal concentrations of cadmium (0.5, 1, 5, and 10 μM) for 96 h. Whole-body cadmium accumulation during this exposure period as quantified using GFAAS (graphite furnace atomic absorption spectroscopy) was found to increase with both exposure length and concentration. Using a transgenic strain of *hsp70/eGFP* (heat shock protein 70/enhanced green fluorescent protein reporter gene) zebrafish, dose-dependent induction of the heat shock response was observed in the olfactory epithelium. Expression of *hsp70/eGFP* in the olfactory epithelium was a highly sensitive biomarker for the effects of cadmium in the olfactory system with a lowest observed effects concentration (LOEC) of 0.5 μM Cd. Strong induction of the transgenic reporter gene correlated closely with cell death (LOEC of 5 μM Cd) and histological alterations (LOEC of 1 μM Cd) in the olfactory epithelium of zebrafish larvae following cadmium exposure. Additionally, loss of sensory cilia from the surface of the olfactory epithelium was observed in larvae exposed to 5 and 10 μM Cd. Furthermore, behaviour tests to assess olfactory function revealed sensory deficits, likely due in part to the cadmium-induced degeneration of the olfactory epithelium ($p<0.05$ for 1 μM; $p<0.001$ for 5 and 10 μM Cd).

To determine if cadmium was entering the cells of the olfactory epithelium by acting as a calcium (Ca) antagonist, zebrafish larvae were co-exposed to 1, 5, or 10 μM Cd with 1 or 5 mM Ca for 96 h. Whole-body cadmium accumulation as quantified using ICP-MS (inductively coupled plasma – mass spectrometry) was decreased in larvae co-exposed to cadmium and

calcium. Additionally, induction of the heat shock response was reduced in the presence of increasing calcium co-treatment. These ameliorating effects of calcium were further revealed in cell death and histological analyses of the olfactory epithelium. Also, larvae co-exposed to cadmium and calcium exhibited greater olfactory sensory function compared to larvae exposed to cadmium only. Significant increases in aversion response were observed in larvae exposed to 5 μM Cd with 1 and 5 mM Ca ($p<0.05$). These results indicate that cadmium gains entry to the olfactory epithelium via calcium uptake systems, wherein it causes damage to the olfactory system and can lead to sensory impairment.

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Dedication

I dedicate this thesis to my parents, Stan and Karen. I may not always say it or act like it, but I appreciate and am grateful for all that you have taught me and provided for me.

Thank you.

I know nothing except the fact of my ignorance.

Socrates (469-399 BCE)

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List of Abbreviations

°C	degrees Celsius
A	anterior
AAS	atomic absorption spectroscopy
ANOVA	analysis of variance
As(III)	arsenite
As(V)	arsenate
ATSDR	Agency for Toxic Substances & Disease Registry
BiP	binding immunoglobulin protein
Ca	calcium
$CaCl_2 \cdot 2\ H_2O$	calcium chloride dihydrate
$CaCO_3$	calcium carbonate
cAMP	3′-5′-cyclic adenosine monophosphate
CAS	chemical abstracts service
Cd	cadmium
$CdCl_2 \cdot 2.5\ H_2O$	cadmium chloride hemi-pentahydrate
Cl	chloride
CNS	central nervous system
Cr	chromium
Cu	copper
D	dorsal
DNA	deoxyribonucleic acid
dUTP	2′-deoxyuridine 5′-triphosphate

EC_{50}	median combined adverse effect concentration
EPA	Environmental Protection Agency
ER	endoplasmic reticulum
eGFP	enhanced green fluorescent protein
g	gram
GFAAS	graphite furnace atomic absorption spectroscopy
GFP	green fluorescent protein
grp75	75 kDa glucose related protein
gro78	78 kDa glucose related protein
h	hour
Hg	mercury
HNO_3	nitric acid
hpf	hours post fertilization
hsc	heat shock cognate
HSE	heat shock response element
HSF	heat shock factor
hsp	heat shock protein
hsp47	47 kDa heat shock protein gene
hsp60	60 kDa heat shock protein
hsp70	70 kDa heat shock protein
hsp70	70 kDa heat shock protein gene or mRNA
hsp70/eGFP	70 kDa heat shock protein gene promoter fused to enhanced green fluorescent protein reporter gene
hsp90	90 kDa heat shock protein

hsp90	90 kDa heat shock protein gene
hsp100	100 kDa heat shock protein
ICP-MS	inductively coupled plasma mass spectrometry
KCl	potassium chloride
KH_2PO_4	monopotassium phosphate
kDa	kilodalton
L	litre
La	lanthanum
LC_{50}	median lethal concentration
L-cys	L-cysteine
LOEC	lowest observed effects concentration
Mg	magnesium
mg	milligram
min	minute
mL	millilitre
µL	microlitre
mM	millimolar
µM	micromolar
mm	millimetre
µm	micrometre
mol	mole
mRNA	messenger ribonucleic acid
mt-hsp70	mitochondrial 70 kDa heat shock protein

N	nitrogen
NaCl	sodium chloride
NaH_2PO_4	monosodium phosphate
ng	nanogram
$NH_4H_2PO_4$	ammonium phosphate monobasic
Ni	nickel
nm	nanometre
P	posterior
p	p-value
Pb	lead
PBS	phosphate buffered saline
PBST	phosphate buffered saline with Tween-20
PCR	polymerase chain reaction
PFA	paraformaldehyde
ppm	parts per million
s	seconds
SEM	scanning electron microscopy
TRIS-HCl	trishydroxymethylaminomethane hydrochloride
TRITC	tetramethylrhodamine isothiocyanate
TUNEL	terminal deoxynucleotidyl transferase-mediated dUT nick end labeling assay
V	ventral
WHO	World Health Organization

Chapter 1

1. Introduction

1.1 General introduction

Cadmium (Cd), a heavy metal with limited biological function (Lane and Morel, 2000), is widely distributed in the environment as a result of natural and anthropogenic activities. Concern over the dispersal of cadmium into the environment has lead to its inclusion on the priority lists of toxic contaminants by the Canadian Environmental Protection Act, the U.S. Environment Protection Agency, and the European Union. Cadmium is a well described developmental toxicant in a variety of vertebrate species including zebrafish, the model system of this research project (Cheng et al., 2000; Blechinger et al., 2002a). Responses to cadmium exposure at the cellular level include activation of heat shock protein synthesis (e.g. Wirth et al., 2002; Souza et al., 2004) and apoptosis (Piechotta et al., 1999; Chan and Cheng, 2003). Furthermore, cadmium toxicity is associated with interference of calcium (Ca) homeostasis (Martelli et al., 2006; Wang et al., 2007), and calcium has been demonstrated to exert protective effects in organisms exposed to cadmium (Hollis et al., 2000; Wu et al, 2007).

Heat shock proteins (hsp) have great potential to be used as biological indicators, or 'biomarkers', of stress. In the heat shock response, hsp's are induced as a part of an adaptive defense mechanism to the presence of denatured proteins in a cell. Because of the connection between expression of hsp's and cellular damage at the protein level, increased expression of hsp's can be correlated to the effects of a wide range of toxicants at the cellular and organism level. Numerous chemical and physical stressors including heat (Lele et al., 1997), metals (Tully et al., 2000; Papaconstantiou et al., 2003), and pesticides (Gupta et al., 2007) are able to induce the heat shock response, which may be an indicator of further effects at the level of the organism.

Traditional toxicology testing has focused on determination of lethal doses and observations of gross effects following maximal exposures. More recently, toxicology testing has focused on lower, more relevant doses and monitoring for slight perturbations in the biochemistry, physiology, or behaviour of the organisms. Coupled with advances in genetic engineering, strains of transgenic organisms with reporter genes under the transcriptional control of inducible *hsp*-gene promoters have been developed. Recently, our laboratory has established an in vivo system that uses activation of *hsp70* expression as a measure of toxicity in the early stages of living larval transgenic zebrafish carrying a stably integrated *hsp70/eGFP* (enhanced green fluorescent protein) reporter gene (Blechinger et al., 2002a). Expression of the reporter gene has proven to be a reliable tissue-specific and dose-dependent indicator of acute cadmium (Blechinger et al., 2002a) and acute arsenic (Salisbury, 2006) toxicities. This established reporter system was used for monitoring the effects of sublethal cadmium exposures in real time, in whole, living zebrafish larvae in this study.

Fish olfactory systems are sensitive to chemical pollutants as the olfactory epithelium is directly exposed the environment. Several studies have shown that olfactory function is impaired after exposure to metals (Julliard et al., 1996; Hansen et al., 1999; Scott et al., 2003); however, little is known as to how waterborne pollutants exert their toxic effects on this tissue. In the environment, properly functioning olfactory systems are of importance for feeding and foraging, reproduction, and predator avoidance. Thus impairment of this sensory system could impair the survival of fish species. For this study, the impacts of sublethal cadmium exposures on larval zebrafish were investigated at the whole organism, cellular, and behavioural level. Additionally, the potential for excess calcium to ameliorate cadmium-induced toxicity in larval zebrafish was also examined.

1.2 Hypotheses

Several hypotheses were addressed in this study:

a) Cadmium from sublethal exposures is accumulated by larval zebrafish in a concentration- and exposure length-dependent manner.

b) Sublethal cadmium exposures have an effect on the olfactory system in developing zebrafish, and that induction of the *hsp70/eGFP* reporter gene in the olfactory epithelium will be indicative of effects on this system at the cellular level, and of sensory impairment altering behaviour.

c) Cadmium accumulation by zebrafish larvae is lessened in the presence of additional calcium.

d) The impact of cadmium on the olfactory system of larval zebrafish is ameliorated in the presence of additional calcium.

1.3 Introduction to cadmium

Physical-chemical properties of cadmium and its environmental fate have been previously reviewed (WHO, 1992a; ATSDR, 2000), and the following is based on these sources unless indicated otherwise. Cadmium (atomic number 48; molecular weight 112.41 g/mol) has only two valence states: Cd^0, the pure metal; and Cd^{2+}, a divalent cation. Pure cadmium exists as a soft, silver-white, lustrous metal, and generally is not found naturally in this valence state. In the environment, the divalent state is prevalent as oxides, carbonates, and sulfides in the ores of other metals, and is commonly found with zinc ores. Cadmium naturally enters the environment as a result of weathering of rocks, forest fires, and volcanic emissions. Anthropogenic sources of cadmium arise mainly from industrial activities and include: burning of fossil fuels; smelting of

ores; application of fertilizers; manufacture of batteries and pigments; electroplating of other metals; and plastics processing. As a result, cadmium is a common pollutant in surface waters where it is found as a hydrated ion ($Cd(H_2O)_6^{2+}$) or in complexes with inorganic or organic ligands. In polluted waters rich in organic compounds, cadmium complexation with these ligands, such as humic acids, has an important role in transport, partitioning, and remobilization of cadmium from sediments and precipitates. Additionally, the solubility of cadmium is influenced by pH, and it is more soluble in acidic waters. Cadmium is removed from the water phase of aquatic systems by precipitation and sorption to sediments, both of which increase with alkalinity.

On the basis of human health concerns, a maximal acceptable contaminant level of 5 µg/L for cadmium in drinking water has been established by Health Canada (1986) and the U.S. Environmental Protection Agency (2002). Most North American drinking water supplies do not exceed 1 µg/L, and as such are below the maximal limit. In surface waters, cadmium concentration is usually less than 0.1 µg/L, but may exceed 1µg/L; while waters near mineral deposits may have cadmium concentrations that exceed 1,000 µg/L. More specifically, two studies by Pyle et al. have reported relatively low cadmium concentrations of 0.01 – 1.96 µg/L (2001) and 0.10 – 2.18 µg/L (2002) in northern Saskatchewan lakes associated with uranium mining. In Oklahoma, a mean cadmium concentration of 0.384 µg/L was measured for a creek that flows through tailing piles remaining from a lead and zinc mine (Yoo and Janz, 2003). In comparison, much higher levels of cadmium contamination of surface waters due to mining and industrial activities have been reported in other parts of the world: 65 – 240 µg/L in Bolivia (Oporto et al., 2007); 0.2 – 401.4 µg/L in India (Srinivasa Gowd and Govil, 2007); and 223 µg/L in South Korea (Lee et al., 2005). These indicate that cadmium contamination of surface waters

is an issue of concern for human and environmental health and in some areas may require remediation.

1.3.1 Toxicokinetics and toxicodynamics of cadmium

Several extensive reviews on the toxicokinetics and toxicodynamics of cadmium have been published (Health Canada, 1986; WHO, 1992a; ATSDR, 2000) and have been used as sources for the following unless indicated otherwise. The only biological function ascribed for cadmium is as an enzyme co-factor in the marine diatom *Thalassiosira weissflogii* (Lane and Morel, 2000). Furthermore, cadmium is not considered a nutrient or an essential metal in animals or humans. In mammals, cadmium in food and drinking water is poorly absorbed in the gastrointestinal tract, with only 5% of cadmium ingested absorbed across the lumen, to the mucosa, and into systemic circulation, while most cadmium that enters the gastrointestinal tract is excreted via the feces. The amount absorbed is influenced by the presence of other metals (e.g. calcium, zinc, iron, and magnesium) and interactions with proteins in the diet. Very little absorption of cadmium occurs through the skin, as dermal absorption is less than 1%, thereby limiting risk potential. In comparison, cadmium is more readily acquired via inhalation, as up to 25% of inhaled cadmium can be absorbed in the alveoli (Klimisch, 1993); however, factors such as size and solubility of inhaled particles dictate the amount of uptake. Inhalational exposure of cadmium is largely associated with smoking and occupational exposures, and not the general population. Inhaled cadmium containing particles that deposit in upper airways are cleared by the mucociliary pathway to the gastrointestinal tract, where low amounts may be absorbed across the lumen. Once it has distributed through the body, cadmium is eliminated very slowly with

relatively equal and small amounts excreted in urine and feces, and has a half-life of 10 to 30 years (reviewed in Jarup, 2002).

Once cadmium enters systemic circulation, the toxicokinetics and toxicodynamics are believed to be similar in mammals and fish. While direct metabolic processes in the body, such as oxidation, reduction, or alkylation, do not convert cadmium into other forms, the divalent cation binds to proteins with anionic groups, especially the sulfhydryl groups of metallothionein and albumin. Upon entry into blood plasma, cadmium binds to albumin and is distributed throughout the body, with the greatest burdens in the liver and kidneys (e.g. Satarug et al., 2002). In the liver, cadmium not bound to metallothionein induces synthesis of the protein as a protective mechanism (reviewed in Nordberg & Nordberg, 2000). Metallothionein is a small cysteine-rich protein capable of binding zinc, cadmium, mercury, and copper, and is the main transport and storage protein for cadmium (Jin et al., 1998). Cadmium bound to metallothionein re-enters the blood stream, is filtered at the glomeruli of the kidney and reabsorbed by the proximal tubule cells. In the tubule cells, the cadmium-metallothionein complex is transported into lysosomes where the complex is catabolized. This releases cadmium from the complex and free cadmium induces metallothionein synthesis in the kidney. When there is an excess of free cadmium in the kidney, damage to the proximal tubules occurs resulting in renal dysfunction (Brzoska et al., 2003) and apoptosis of kidney cells (Ishido et al., 1995).

The health effects of cadmium in mammals are varied and dependent on route of exposure and dose. Acute inhalation exposure results in pulmonary edema and pneumonitis associated with destruction of mucus membranes, which may be fatal (Godt et al., 2006). Chronic occupational inhalation of cadmium can result in obstructive lung disease and emphysema. Ingestion of large amounts of cadmium causes acute gastrointestinal irritation,

causing vomiting and diarrhea (Godt et al., 2006). With chronic cadmium ingestion, the kidney is a main target organ, resulting in tubular dysfunction and subsequent proteinuria. Other health effects associated with cadmium exposure include osteomalacia and osteoporosis, which are attributed to cadmium-induced hypocalcemia, and carcinogenesis.

1.3.2 Cadmium uptake and accumulation in fish

Cadmium is acutely toxic to fish and other aquatic organisms, resulting in mortality at lower concentrations than observed with other metals. This toxicity is attributed to the ability of cadmium to act as a calcium antagonist to gain entry into the body and subsequent disruption of calcium homeostasis (Verbost et al., 1987, 1988, 1989). Similar to mammals, absorbed cadmium readily bioaccumulates in fish due to its slow rate of elimination. The three main routes of cadmium uptake in fish are through the gills, gastrointestinal tract, and olfactory rosette (Scott et al., 2003). Acquisition of cadmium from the surrounding waters occurs primarily at the gill, where Cd^{2+} readily enters the gill epithelium via the lanthanum (La^{3+})-sensitive apical Ca^{2+} channels (Verbost et al., 1987, 1989; Wicklund Glynn et al., 1994). Absorption of cadmium from dietary sources, like gill uptake, occurs through nonselective calcium transport systems in the gastrointestinal tract (Schoenmakers et al., 1992). Finally, cadmium can accumulate in fish olfactory systems following water borne exposures (Tjalve et al., 1986; Scott et al., 2003).

Following gill and gastrointestinal uptake, cadmium accumulates primarily at the sites of entry, as well as the liver and kidneys. Waterborne sublethal exposures resulted in the greatest accumulation in the kidneys followed by liver and gills in several juvenile fish species (de Conto Cinier et al., 1997; Melgar et al., 1997; De Smet et al., 2001; Hollis et al., 2001). Adult rainbow trout exposed to dietary cadmium had the highest level of accumulation in the gut (i.e. the point

of entry; Chowdhury et al., 2004). Very little to no cadmium accumulates in the muscle tissue after waterborne or dietary exposure, suggesting that the accumulation limits of the kidney and liver must be saturated before muscle is used as a sink (de Conto Cinier et al., 1997; Chowdhury et al., 2004).

Accumulation of cadmium is also found in the olfactory system, including the olfactory rosette, olfactory nerve and olfactory bulb in the brain following waterborne exposure (Tjalve et al., 1986). Many other metals including manganese, mercury and nickel have also been shown to accumulate in the olfactory system (reviewed in Tjalve and Henrikkson, 1999). Cadmium is taken up by the nerve cells in the olfactory rosette and transported along the axons of the primary olfactory nerve to their terminations in the olfactory bulb (Gottofrey and Tjalve, 1991). Cadmium exposure induces metallothionein production in the primary olfactory nerve and transport of the metal along the axons is as a Cd-metallothionein complex (Tallkvist et al., 2002). Accumulated cadmium in the brain is confined to the anterior part of the olfactory bulb, suggesting that cadmium does not exit the primary olfactory neurons (Tjalve and Henriksson, 1999).

Studies involving embryonic and larval fish have measured cadmium acquisition at the level of the whole embryo or larva (i.e. are not tissue specific). Cadmium uptake by Atlantic salmon embryos was rapid until a dose-dependent saturation level was reached; however, the cadmium content in the newly hatched alevins was much lower indicating that the metal was associated with the membranes of the chorion and not the developing embryo (Rombough and Garside, 1982). Rates of cadmium accumulation by newly hatched larval Atlantic salmon (Rombough and Garside, 1982) and tilapia (Chang et al., 1998) exposed to concentrations above 1 μg/L was independent of treatment concentration, indicating that sites of entry were saturated.

A recent study of cadmium accumulation into zebrafish eggs reported that metal uptake increased with exposure concentration and length of exposure period; furthermore, most of the cadmium was associated with the chorion and only 1% with the embryo (Burnison et al., 2006).

1.3.3 Influence of calcium on cadmium uptake and accumulation in fish

A competitive interaction between cadmium and calcium for uptake in fish, leading to reduced cadmium accumulation, has been demonstrated by numerous authors (e.g. Chang et al., 1997; Hollis et al., 2000; Wu et al., 2007; Zhang and Wang, 2007). Central to this competition is that waterborne cadmium uptake at the gills, the main site of cadmium uptake, occurs via lanthanum-sensitive, voltage-independent calcium channels (Verbost et al., 1987, 1988). Increased waterborne calcium has been demonstrated to inhibit waterborne cadmium uptake (Wicklund Glynn et al., 1994; Niyogi and Wood, 2004), emphasizing the antagonistic interaction at calcium uptake channels. Additionally, use of calcium channel blockers effectively reduced waterborne cadmium uptake in different fish species (Wicklund Glynn, et al., 1994; Zhang and Wang, 2007). Along with reducing cadmium uptake, excess calcium was found to limit the toxic effects of cadmium, including reducing the extent of growth inhibition in tilapia larvae (Wu et al., 2007), and ameliorating cadmium-induced plasma hypocalcemia (Pratap et al., 1989). These studies demonstrate the protective effects that excess waterborne calcium can have on fish exposed to waterborne cadmium. Additionally, several studies have demonstrated that dietary calcium can also limit waterborne cadmium uptake. Combining chronic waterborne cadmium exposures with dietary calcium supplementation, Zohouri et al. (2001), Baldisserotto et al. (2004), and Franklin et al. (2005) observed greatly reduced cadmium accumulation in rainbow trout and a reduction in calcium uptake via the gills. A further study suggested that this observed

decrease of cadmium and calcium influx at the gills, when fish are fed a calcium-enriched diet, was due to decreased expression of a gill epithelial calcium channel (Galvez et al., 2007); a reduction in calcium channels in the gill epithelium would limit uptake of both cadmium and calcium. Together, these observations support the model that a primary effect of cadmium exposure is limiting calcium uptake via the gills, and secondary effects of cadmium toxicity are often the result of altered calcium homeostasis.

1.3.4 Effects of cadmium on zebrafish

Several studies have described the various effects of cadmium on zebrafish, including morphological alterations in gill tissue (Karlsson-Norrgren et al., 1985), changes in gene expression (Gonzalez et al., 2006), developmental malformations (Cheng et al., 2000; Cheng et al., 2001; Chow and Cheng, 2003), cell death (Chan and Cheng, 2003), and stress responses (Blechinger et al., 2002a; Hallare et al., 2005a; Chen et al., 2007). Karlsson-Norrgren et al. (1985) examined the impact of chronic cadmium exposures in zebrafish gills, as this tissue is the main site of waterborne cadmium uptake (see section 1.3.2). Cadmium exposure resulted in disorganization of the gill lamellae, lesions from swelling and degeneration of gill epithelia, and loss of microvilli associated with chloride cells; these adverse effects would likely impair the essential functions of gas exchange and osmoregulation. A study of gene expression following short- and long-term cadmium exposures found that strong *hsp70* gene expression in the gill tissue was present after a 7 day exposure, while after 21 days *hsp70* expression had returned to normal levels and metallothionein expression was induced (Gonzalez et al., 2006). This suggests that the heat shock response was a short-term adaptation to cadmium exposure and metallothionein was likely utilized for long-term detoxification and sequestering of cadmium.

Similarly in developing zebrafish, induction of *hsp70* expression in the gills of larvae was found to be present after only a short, 3 hour pulse exposure (Blechinger et al., 2002a), while metallothionein expression was only detected after 96 and 120 hour length exposures (Chen et al., 2007).

Interestingly, cadmium exposure during development can disrupt normal muscle formation (Cheng et al., 2000; Chow and Cheng, 2003), yet does not accumulate in muscles of adult zebrafish (Gonzalez et al., 2006). Presently, there are no reports on cadmium levels specifically in muscle tissue of developing zebrafish; however, no metallothionein expression was evident in muscle of zebrafish larvae exposed to cadmium for up to 120 hours (Chen et al., 2007), further suggesting that cadmium is not present or stored in the muscle to a great extent. Exposure to cadmium during development resulting in tail and trunk malformations, and abnormal tail curvature has been reported by several authors (Cheng et al., 2000; Blechinger et al., 2002a; Chan and Cheng, 2003; Chow and Cheng, 2003; Hallare et al., 2005a). Suggested cellular and molecular bases for these gross abnormalities include reduced myosin heavy-chain production (Cheng et al., 2000), altered somite formation, loss of muscle fibers, (Chow and Cheng, 2003), and ectopic apoptosis in the regions of malformation (Chan and Cheng, 2003).

1.4 Introduction to the olfactory system

Like the other senses, the olfactory system allows an organism to gain information regarding its surroundings by perception of specific cues. With the sense of smell, these are chemical cues present in the environment that interact with olfactory sensory neurons to generate a nerve impulse, a process referred to as olfactory chemoreception (Farbman, 1992). Since their aquatic environment alters light and sound cues, fish have an increased reliance on the

chemosensory systems of olfaction, gustation, and solitary chemosensory receptors (Hara, 1992a). Of these chemosensory systems, the olfactory system is considered crucial as it has been implicated in many essential behaviours (Whitlock, 2003). Additionally, the olfactory system is of toxicological importance as it is directly exposed to external environment and any pollutants or hazardous compounds found within an organism's surroundings. Furthermore, alterations in normal behaviours are important endpoints for toxicological assessment as behaviour is an observable link between the physiology of an organism and its environment (Scott and Sloman, 2004).

1.4.1 Anatomy and organization of the fish olfactory system

The primary olfactory pathway consists of bipolar sensory neurons with dendrites exposed to the environment at the periphery and long axons forming the olfactory nerve which synapse at the olfactory bulb. This organization is similar for all animals, vertebrates and invertebrates, with modifications between divergent species (Farbman, 1992). Specifically, the morphology and location of the fish olfactory system is diverse between the different classes of fish (i.e. jawless, cartilaginous, and bony fishes). As zebrafish, the model organism utilized in this study, are bony fish (or teleosts) the following will focus on the anatomy of this classification.

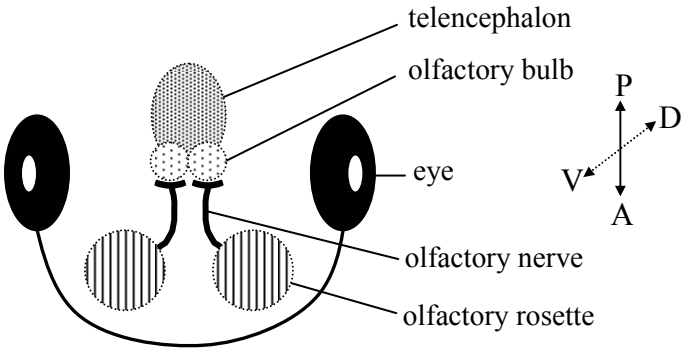
In teleosts, paired olfactory organs are located on the dorsal surface of the fish, above and to the sides of the mouth, and anterior to the eyes (reviewed in Laberge and Hara, 2001). In adult fish, each olfactory organ is located in a cavity between two openings, or nares, through which water flows during swimming. In adult zebrafish, the peripheral olfactory organ within the cavity has multi-lamellae attached to a midline raphe, though the morphology and

arrangement of the lamellae is diverse between teleosts (Hansen and Zielinski, 2005). Sensory and non-sensory cells forming the olfactory epithelium line the lamellae, and since lamellae are often organized to form a circular structure, the peripheral olfactory organ is commonly referred to as the olfactory rosette. The sensory cells, also referred to as olfactory receptor neurons, are bipolar neurons with their cell bodies arranged in layers through the olfactory epithelium (Zeiske et al., 1992). Each olfactory receptor neuron extends a dendrite to the surface and proximally projects an axon. The millions of axons are bundled to form the olfactory nerve and synapse with secondary olfactory neurons of the olfactory bulb, a central nervous system (CNS) structure (Laberge and Hara, 2001). At the olfactory bulb, sensory neuron axon terminals and dendritic ends of olfactory bulb neurons form spherical clusters termed glomeruli (Laberge and Hara, 2001). Furthermore, each sensory neuron axon only terminates at one glomerulus, and each glomerulus receives nerve impulses from a small number of sensory neurons (Hara, 1992b). Schematic illustrations of the organization of the fish olfactory system are presented in Figure 1.1, panels A and B.

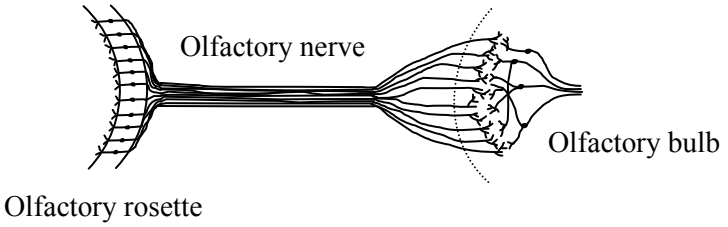
Three types of sensory neurons are present in the peripheral olfactory organ of zebrafish, and are named for the projections from their dendritic ends: ciliated, microvillous, and crypt cells, which have both cilia and microvilli (Hansen and Zeiske, 1998). This terminal end of a dendrite is referred to as an olfactory knob. In adult zebrafish the olfactory knob of ciliated cells is pronounced and bears 3 to 7 cilia, ~2-3 μm long and ~0.25 μm in diameter, while the olfactory knob of microvillous cells is less pronounced and bears 10 to 30 short microvilli that are 0.5-0.8 μm in length (Hansen and Zeiske, 1998). Relative to ciliated and microvillous cells, very few crypt cells are present in the olfactory epithelium and were often unnoticed or overlooked in early examinations of fish olfactory epithelium (Hansen and Zielinski, 2005). These

Figure 1.1 Schematic illustrations of the fish olfactory system. Panel A portrays the anatomical location and organization of the components of the olfactory system. Panel B depicts the sensory neurons with their cell bodies in the epithelium, the axons forming the olfactory nerve and extending to the olfactory bulb to synapse with secondary olfactory neurons.

A


telencephalon
olfactory bulb
eye
olfactory nerve
olfactory rosette
P
D
V
A

B


Olfactory nerve
Olfactory bulb
Olfactory rosette

morphologies of sensory neurons also have biochemical and functional differences as they express different types of odorant receptors and respond to different odorants (Hansen and Zielinski, 2005). Additionally, axons from ciliated and microvillous sensory neurons project to mutually exclusive glomeruli in the olfactory bulb (Sato et al., 2005), further suggesting a functional difference between the types of cells.

Also present in the olfactory epithelium are several types of non-sensory cells including supporting cells, basal cells, ciliated non-sensory cells, and goblet cells. Supporting cells are located throughout the sensory epithelium and surround, separate, and provide structural support for the receptor cells (Zeiske et al., 1992). Basal cells are the smallest of the cells in the olfactory epithelium and are scattered along the base of this tissue (Zeiske et al., 1992). These cells act as stem cells in the olfactory epithelium allowing for growth and regeneration of olfactory sensory neurons, and may also differentiate into supporting cells (Hansen and Zielinski, 2005). Ciliated non-sensory cells extend through the entire sensory epithelium and bear numerous cilia on the epithelial surface (Zeiske et al., 1992). In adult zebrafish, up to 60 cilia, 7-8 μm long, are located on each ciliated non-sensory cell (Hansen and Zeiske, 1998). These long cilia are referred to as kinocilia as they are involved in moving water and mucus across the epithelia surface via synchronous beating of the cilia (Hansen and Zielinski, 2005). Goblet cells secrete mucopolysaccharides at the surface of the olfactory epithelium forming a mucus layer (Zeiske et al., 1992).

1.4.2 Olfactory chemoreception

Olfactory perception and discrimination begins when compounds enter the peripheral olfactory organ and bind to specific odorant receptors on the cilia or microvilli on the dendritic

ends of olfactory sensory neurons. Binding to odorant receptors causes G-protein activation of adenylate cyclase resulting in production of cyclic AMP (cAMP; Jones and Reed, 1989). Elevated intracellular cAMP causes cyclic-nucleotide-gated channels in the plasma membrane to open (Nakamura and Gold, 1987). This results in an influx of calcium and sodium ions (Trombley and Westbrook, 1991) and opening of a Ca^{2+}-gated Cl^- channel (Kurahashi and Yau, 1993; Lowe and Gold, 1993). The influx of cations and efflux of chloride anions causes depolarization of the plasma membrane which ultimately leads to an action potential and transmission of the signal to the olfactory bulb for higher processing and integration (Laberge and Hara, 2001).

The ability to detect and distinguish odorants lies in the odorant receptors. Each olfactory sensory neuron expresses one specific odorant receptor and the axons of sensory neurons with identical receptors converge in the same glomerulus in the olfactory bulb (reviewed in Mombaerts, 1999; Nakamura, 2000). A given odorant can activate several receptors and each receptor can bind multiple odorants. Discrimination between odorants is achieved as different odorants interact with different groups of receptors (Malnic et al., 1999). Genes that encode odorant receptors were first identified as a large multigene family of G-protein coupled receptor protein receptors that were selectively expressed in the olfactory sensory neurons (Buck and Axel, 1991). For this work, Linda Buck and Richard Axel received the Nobel Prize in 2004. Since the original publication, candidate odorant receptor genes have been identified for many species, including zebrafish, based on sequence homologies (e.g. Byrd et al., 1995), and this remains an active area of research. Recently, 143 odorant receptor genes organized in genomic clusters were identified in the zebrafish genome and grouped into 8 gene families based on amino acid identity (Alioto and Ngai, 2005). The different combinations of odorant receptor

activation and the large number of odorant receptors allows for the detection and differentiation of scores of compounds.

1.4.3 Development of the zebrafish olfactory system

The primary olfactory system develops from paired ectodermal thickenings, termed olfactory placodes, on the head of embryonic zebrafish. These placodes eventually give rise to olfactory sensory neurons, basal cells, and support cells (Hansen and Zeiske, 1993). At 15-17 hour post fertilization (hpf), the beginnings of the olfactory placodes are detectable as a loose aggregation of cells that have separated from the neural plate, just below the epidermis on the head, adjacent to the developing brain and eyes (Hansen and Zeiske, 1993; Whitlock and Westerfield, 1998, 2000). Convergence of cells from the anterior neural plate forms the closely packed placodes filling the pared spaces between the developing eyes and brain (Hansen and Zeiske, 1993; Whitlock and Westerfield, 2000). At 24 hpf, dendrite-less pioneer neurons emerge from the placodes and initiate connections with the developing telencephalon (Whitlock and Westerfield, 1998). Once these connections are made, cells of the placode begin to elongate, extending the length of the placode, and each naris is opened by 32 hpf as the overlying epidermis separates from the placodes (Hansen and Zeiske, 1993). The olfactory sensory neurons form a second wave of neuron growth, as thick bundles of axons extend toward the developing brain following the paths of the pioneer neurons, and cilia and microvilli emerge from the olfactory pit (Hansen and Zeiske, 1993; Whitlock and Westerfield, 1998). Once the sensory neurons reach the developing olfactory bulb in the CNS, the pioneer neurons undergo apoptosis at ~48 hpf (Whitlock and Westerfield, 1998). The tracts set by the pioneer neurons are essential as ablation of the pioneer neurons prior to growth and extension of their axons towards

the brain results in misrouting of sensory neuron axons (Whitlock and Westerfield, 1998). After entering the CNS, the sensory neuron axons diverge and segregate to form glomeruli in the developing olfactory bulb (Dynes and Ngai, 1998).

Concurrently with the events of sensory neuron growth and development described above, the olfactory pits continue to grow and the different cell types are established. At 48 hpf, ciliated and microvillar receptor neurons, support cells, basal cells, and non-sensory ciliated cells are observable (Hansen and Zeiske, 1993). When the zebrafish hatches from its chorion around 72 hpf, the olfactory pits are round openings covered in receptor cilia and microvilli, and bordered by long kinocilia of the non-sensory ciliated cells that are densely arranged around the edges of the olfactory pits (Hansen and Zeiske, 1993). As the larvae grow in the weeks following hatch, the olfactory pits elongate to a more oval shape and the olfactory epithelium begins to fold forming the midline raphe and first lamella (Hansen and Zeiske, 1993). About one month post fertilization, lateral extensions from the sides of the olfactory pit slowly begin to grow toward each other forming an isthmus above the pit, creating incurrent and excurrent nares (Hansen and Zeiske, 1993).

As the olfactory sensory neurons are forming, the cells begin expressing the genes necessary for chemosensory functionality. By 24 hpf, expression of cyclic nucleotide-gated channels was observed in a significant area of each olfactory placode (Barth et al., 1996). Additionally, two research groups have detected expression of odorant receptors in the olfactory placodes as early as 30 hpf, corresponding with the opening of each naris (Barth et al., 1996; Byrd et al., 1996). However odorant receptor gene expression observed in both studies was weak, and not all genes tested were expressed at this time. From about 36 hpf to hatching at 72 hpf, expression of odorant receptors increased as more genes were expressed and in greater

relative amounts, indicating a differential onset of odorant gene expression (Barth et al., 1996; Byrd et al., 1996). Furthermore, zebrafish larvae exhibit behavioural responses to amino acids, known olfactory cues, at three to four days post fertilization (Lindsay and Vogt, 2004; Vitebsky et al., 2005) suggesting that zebrafish have a functioning olfactory system shortly after hatching. However, olfactory function is not complete at this early age as developing zebrafish did not show responsiveness to all odorants tested until four to five weeks post fertilization (Vitebsky et al., 2005). Also, Byrd et al. (1996) observed that the pattern of gene expression of specific odorant receptors was not similar to that of mature adults until five weeks post fertilization. Together, these studies indicate that zebrafish have some olfactory perception as young larvae, but that complete functionality is not achieved until weeks later in the juvenile stage, potentially corresponding with morphological maturity (no study at present has directly examined this).

1.4.4 Olfactory-mediated behaviours in fish

The olfactory system has a central role in many fish behaviours, including feeding, kin recognition, reproduction, homing, and predator avoidance. These different behaviours are essential for individual and population survival attesting to the importance of this chemosensory system. Direct evidence of the primary importance of the olfactory system in feeding has been observed using experimentally induced anosmic fish (i.e. fish without a sense of smell). Sturgeon were unresponsive to food odors after cauterization of both olfactory rosettes, while sturgeon with only one rosette cauterized responded at the same level of sensitivity as control fish (Kasumyan, 2002). Also, feeding responses to amino acid stimuli were not observed after transection of the olfactory nerves in herring larvae (Dempsey, 1978) and removal of the peripheral olfactory organs in adult rainbow trout (Valentincic and Caprio, 1997).

Kin recognition refers to an organism's differential treatment of conspecifics based on the degree of relatedness, likely based on perception of pheromones released by each organism (Olsen, 1992). Studies using fish have demonstrated that several degrees of relatedness can be distinguished by fish, including between familiar and unfamiliar kin, kin and non-kin, different shoals, foreign populations, and different species (Gerlach and Lysiak, 2006; Behrmann-Godel et al., 2006). These studies utilized holding tank water as odor stimuli to assess fish preferences for one group over another, as the pheromones responsible for kin recognition were assumed to have been released into the water by the fish held in the tanks, likely via urine (Moore et al., 1994). Correspondingly, Atlantic salmon had electrophysiological olfactory responses to urine from siblings and non-kin, though greater responses were observed with kin (Moore et al., 1994), suggesting that cues to relatedness may be present in urine.

Different reproductive behaviours in fish are influenced by pheromones including mate selection, inbreeding avoidance, and spawning. For example, female cichlids did not display a preference for mates when given only visual cues, but strongly preferred to mate with a conspecific male over a second male of a closely-related sympatric species when given olfactory and visual cues (Plenderleith et al., 2005). Also, at sexual maturity female zebrafish exhibited a reverse in odor preferences, choosing odor from unrelated males over male kin, potentially as a means to prevent inbreeding (Gerlach and Lysiak, 2006). During spawning, the temporal release of several pheromones induces changes in behaviour and physiology of other fish to increase breeding success (reviewed in Kobayashi et al., 2002). More specifically, pheromones released by pre-ovulatory female goldfish stimulate increased male-female interactions, increased sperm production, and synchronous ovulation by other females; and, a prostaglandin pheromone released by post-ovulatory females attract males and induces courtship behaviours (Kobayashi et

al., 2002). Additionally, experimentally induced anosmic males had fewer interactions with females and often failed to spawn, emphasizing the role of the olfactory system in pheromone perception and reproduction (Kobayashi et al., 2002).

The return of fish to a home territory, range, or river, and the role of the olfactory cues in these homing behaviours are well described. Salmon are well known to return to their natal rivers from the ocean for spawning. This behaviour has been strongly associated with olfactory imprinting, as juvenile salmon learn the scents of their home stream and use these memories to return as adults (reviewed in Dittman and Quinn, 1996). A predominant role for olfactory perception in homing behaviours of other fish has also been reported, as fewer experimentally induced anosmic black rockfish returned to their home habitat (Mitamura et al., 2005), and no preference for water from home reefs was found in anosmic coral reef fish (Doving et al., 2006). Additionally, larval reef fish displayed a strong preference for water from home reefs (Gerlach et al., 2007), indicating that perception and imprinting of home scents occurs during fish development.

Assessment of predation risk by fish is partially based on olfactory-dependent detection of a pheromone, termed alarm substance. Alarm substance is produced by specialized epidermal club cells known as alarm cells (reviewed in Chivers and Smith, 1998). This pheromone is only released when the skin is mechanically damaged, such as by a predator attack (Wisenden, 2000). Detection of alarm substance by conspecifics induces stereotypical behavioural responses including increased shelter use, decreased activity or freezing, schooling, and rapid escape response (Chivers and Smith, 1998). Additionally, avoidance behaviour has been observed in fish exposed to holding waters from predatory fish (McLean et al., 2007) suggesting that assessment of predation risk involves multiple olfactory stimuli.

1.4.5 Environmental pollutants and fish olfactory systems

Fish olfactory systems are especially vulnerable to water borne pollution because the olfactory epithelium is continuously exposed to the surrounding water and any toxicants present in the water. The impact of environmental pollutants on fish olfactory systems is an active area of research, and effects of some pollutants, especially metals and pesticides, are well described (e.g. Sandahl et al., 2007; Tierney et al., 2007a). Additionally, several metals, including cadmium, mercury, manganese and nickel, have been demonstrated to enter the olfactory epithelium and are subsequently transported to the olfactory bulb and CNS, effectively bypassing the blood-brain-barrier (Tjalve and Henrikkson, 1999). Several previous studies have reported histological changes and fine structural alterations to cilia of the olfactory epithelium of fish following waterborne exposure to cadmium (Stromberg et al., 1983; Hernadi, 1993), copper (Julliard et al., 1993; Julliard et al., 1996; Hansen et al., 1999), and mercury (de Oliveira Ribeiro et al., 2002). Furthermore, waterborne exposures to cadmium (Baker and Montgomery, 2001; Scott et al., 2003) and copper (Saucier et al., 1991; Beyers and Farmer, 2001; Carreau and Pyle, 2005; Sandahl et al., 2007) had effects on normal behavioural responses to olfactory stimuli, including decreased response to alarm substance and reduced kin recognition. Additionally, copper was found to decrease electrophysiological responses in the olfactory epithelium to amino acids and bile salts, both known odorants, within ten minutes of exposure to copper (Baldwin et al., 2003). Together these studies indicate that metals have a direct impact on sensory cells of the olfactory epithelium, and can lead to functional impairment of the olfactory system even after very short exposures.

Pesticides are widely used chemicals that readily enter aquatic ecosystems via direct application and/or run-off from land use, and several pesticides have been demonstrated to impair olfactory function in several fish species (e.g. Scholz et al., 2000). For example, male Atlantic salmon exposed to diazinon, an organophosphate insecticide, had reduced electrophysiological and physiological responses to prostaglandin (i.e. the pheromone released by post-ovulatory females; Moore and Waring, 1996). Diazinon exposure in chinook salmon was also found to reduce responses to alarm substance and homing behaviours (Scholz et al., 2000). Because the potential effects of diazinon on the olfactory epithelium were not examined, this observed sensory impairment may be due the neuronal acetylcholinesterase activity of diazinon and/or direct, non-specific effects on sensory cells. In additional studies, several herbicides and a carbamate insecticide reduced electrophysiological responses by olfactory sensory neurons to amino acids and bile salts, in trout and salmon (Tierney et al., 2007a, 2007b), indicating that pesticides may exert direct effects on the olfactory sensory neurons. As these pollutants can impact several behaviours crucial for fish survival and reproductive success, at environmentally relevant concentrations, there is potential for long-term effects on success of fish populations.

1.5 Introduction to heat shock proteins

Heat shock proteins (hsp) are a family of evolutionarily conserved proteins expressed by prokaryotes and eukaryotes in response to heat and other stressors. Ritossa (1962) first discovered hsp's when he observed puffs along the polytene chromosomes of the fruit fly *Drosophila melanogaster* in response to severe heat shock. The puffs corresponded to areas of active transcription of genes encoding the newly discovered hsp's. Today hsp's are known to act

as molecular chaperones, are inducible by a wide range of stressors, and are often referred to as stress proteins to reflect this more general role.

Different families of heat shock proteins are distinguished from each other based on relative molecular mass: hsp100, hsp90, hsp70, hsp60, and the low molecular weight hsp's from 16 to 30 kDa (reviewed in Iwama et al., 1998). Hsp's are located in many cell compartments including the cytosol, endoplasmic reticulum (ER), mitochondria and chloroplasts (Sanders, 1993). Although often referred to as stress proteins, some members of the hsp families are constitutively expressed and are referred to as heat shock cognates (hsc). During unstressed or normal conditions, hsc's function as molecular chaperones to direct the folding and assembly of proteins, and preventing improper protein-protein interactions (Ryan and Hightower, 1999). When stress to cells results in damage to proteins, a condition referred to as proteotoxicity, expression of hsp's is induced to provide additional chaperoning to repair denatured proteins and protect against future damage. For the purpose of this thesis, hsp will be used to refer to both the constitutively expressed and stress-inducible proteins.

1.5.1 The heat shock response: induction and regulation

The heat shock response is induced by proteotoxic stressors and marked by an accumulation of hsp's. Expression of the inducible hsp's is tightly regulated at the level of transcription by heat shock transcription factors (HSF's). While a single HSF gene exists in yeast, several HSF's have been identified in vertebrates. The existence of multiple HSF's (HSF1-4) suggests that HSF's have different roles in the stress response and normal physiology (Pirkkala et al., 2001). Analogous to the single HSF found in yeast and the fruit fly, the

vertebrate HSF1 is the activator of heat shock gene expression in response to physical and chemical stress.

A model for the regulation of the heat shock response has been proposed and extensively reviewed (i.e. Morimoto et al., 1992; Iwama et al., 1998; Katschinki, 2004). In the cytosol, HSF1 is present as an inactive, monomeric protein bound to hsp70 under non-stress conditions. Following a proteotoxic event, hsp70 and other chaperones are recruited to aid the repair or destruction of denatured proteins. Free HSF1 translocates to the nucleus where it forms homotrimers capable of binding DNA. Trimeric HSF1 binds to DNA at a specific, conserved sequence termed the heat shock response element (HSE), which is an element of heat shock gene promoters. Binding of HSF1 trimers to the HSE's of heat shock protein promoter regions is not sufficient to activate transcription until phosphorylation of HSF1 at specific serine residues. Phosphorylated HSF1 trimers activate the transcription of *hsp70* to meet the chaperoning needs required by the cell. Once excess hsp70 accumulates, it binds to HSF1 triggering release from the DNA, dissociation back to the monomeric state, and the hsp70-HSF1 heterodimers return to the cytosol. Integral to the regulation of the heat shock response is the equilibrium between binding of hsp70 to HSF1 and to denatured proteins. Experimental evidence has indicated that hsp90 is also central to the regulation of HSF1 and control of the heat shock response (Ali et al., 1998).

1.5.2 Heat shock protein 70 (hsp70) family

Members of the large hsp70 family regulate protein folding and translocation in many parts of the cell. Hsp70 and its cognates have many functions within a cell including: a role as molecular chaperones interacting with nascent polypeptides to ensure proper folding;

maintaining proteins in an intermediate configuration for targeting and translocation to organelles; and, repairing or removing denatured proteins in the cytosol and nucleus (Sander, 1993). Mitochondrial mt-hsp70 or grp75 (named as a glucose-regulated protein) is constitutively expressed and is responsible for translocation of proteins across the mitochondrial membranes (Ryan and Hightower, 1999). Also constitutively expressed, grp78 or BiP (binding immunoglobulin protein) is located in the lumen of the ER and is involved with import of proteins to the ER and assists in their folding (Ryan and Hightower, 1999). Unlike other members of the heat shock family, grp78 is strongly induced by glucose deprivation, not heat shock.

1.5.3 Heat shock protein 70 as a biomarker

Because they have been extensively studied and are highly conserved across diverse phyla, hsp70 family member have great potential to be used as a biomarker of toxic stress. Hsp70 is an excellent candidate as a biomarker of exposure because the heat shock response is induced by a variety of environmental stressors. A selection of recent studies that have utilized hsp70, either protein levels or gene expression, is presented in Table 1.1.

Table 1.1 A sample of studies utilizing hsp70 as a biomarker

Stressor	Organism	Reference
Cd^{2+}	Zebrafish	Blechinger et al., 2002a, 2007 Hallare et al., 2005a
Cu^{2+}, As(III)	Zebrafish	Seok et al., 2006, 2007
Cd^{2+}, Pb^{2+}, Cr^{6+}	Sea bream blood cells	Fulladosa et al., 2006
Hg^{2+}, Cd^{2+}, As(III)	Chicken embryos	Papaconstantinou et al., 2003
Cd^{2+}, Pb^{2+}, Cu^{2+}, Hg^{2+}	Earthworm	Nadeau et al., 2001
As(III), As(V), arsenicals	Bay mussel	La Porte, 2005
Cd^{2+}, Cu^{2+}, Ni^{2+}, organochlorines	Black sea bream fibroblasts	Deane and Woo, 2006
heat/cold shock, Cd^{2+}, Cu^{2+}, Pb^{2+}	Cultured cabbage armyworm cells	Sonoda et al., 2007
Cu^{2+}, esfenvalerate	Striped bass	Geist et al., 2007
heat shock, osmostic stress, Cd^{2+}	Marine macroalga and duckweed	Ireland et al., 2004
acid stress, heat shock, Cd^{2+}	Sea urchin coelomocytes	Matranga et al., 2002
nonylphenol, 17β-estradiol, β-naphthoflavone	Black goby	Maradonna and Carnevali, 2007
Pb and Zn mining effluent	Black bullhead and bluegill sunfish	Yoo and Janz, 2003
mariculture, industrial, and urban runoff	Marine mussel	Hamer et al., 2004
ammonia, polyaromatic hydrocarbons, pentachlorophenol	Brown trout	Luckenbach et al., 2003
13 different industrial wastes	HeLa cell culture	Ait-Aissa et al., 2003

Although not exhaustive, this compilation is indicative to the broad range of stressors and the biological diversity of organisms and model systems to which this biomarker has been applied. Hsp70 is also a potential biomarker of effect because increases in expression can be directly related to adverse proteotoxic effects at the cellular level and organism level (reviewed in Bierkens, 2000). Additionally, the ubiquity and consistency with which hsp70 is expressed in response to a broad spectrum of stressors makes it an appealing biomarker for laboratory and field studies. Furthermore, a stressor-induced heat shock response can occur in the absence of

overt effects, including mortality, in the exposed organisms (Hallare et al., 2005b). This indicates that induction of *hsp70* expression is a more sensitive endpoint than traditional toxicological endpoints and has great potential to be used as a biomarker in an integrated approach (i.e. evaluations at multiple levels of biological organization) to environmental biomonitoring (Bierkens, 2000).

1.6 Zebrafish as a model system

The zebrafish, *Danio rerio*, is a small tropical aquarium fish that is a popular vertebrate model system for developmental biologists and toxicologists (e.g. Fraysse et al., 2006). Many features make this organism an attractive choice for researchers. The adult fish are small, inexpensive, hardy, and easy to maintain. Zebrafish have the potential to produce large numbers of offspring allowing investigators to obtain large sample sizes in a short period of time. The rapid development of the embryos (from fertilization to hatch in 3 days) has been well characterized (Kimmel et al., 1995). Because fertilization is external, embryos can be readily collected for experiments and exposure to chemicals is easily achieved placing embryos/larvae into water containing the compound(s) of interest. The relatively large size and transparency of the developing fish is advantageous for observations of all cells. Additionally, many resources are available for genetic studies of zebrafish development and the complete genome sequence is available.

With advancements in DNA manipulation and recombination technologies, transgenic zebrafish emerged as invaluable tools to study gene expression. (The creation of transgenic organisms is described in section 1.7). An array of stable transgenic lines of zebrafish have been and continue to be created in which genes encoding fluorescent reporter proteins, such as green

fluorescent protein (GFP), are linked with tissue-specific or inducible promoters (Udvadia and Linney, 2003). These transgenic reporter systems have great utility in monitoring of cell movements and gene expression during development, as these activities are observable in living embryos and larvae. For example, using the myeloperoxidase promoter to drive GFP expression in zebrafish leukocytes, Mathias et al. (2006) observed leukocyte chemotaxis to the site of an injury and subsequent movement of leukocytes from the injury back to the vasculature; this retrograde chemotaxis was proposed as a novel mechanism to resolve inflammation after injury. Also, selective expression of GFP in melanophores of zebrafish was achieved using a promoter for a melanin synthesis enzyme (Zou et al., 2006); furthermore, the specific labeling of pigment cells allowed for ready isolation of these cells using flow cytometry. These examples help to illustrate some of the advantages of transgenic zebrafish, such as observation of previously undescribed events and identification and isolation of specific cell populations.

1.7 Transgenic organisms as biosensor and biomarkers

Traditional methods for detection of gene expression include *in situ* hybridization for mRNA transcripts and immunohistochemical staining for protein products. Both of these techniques require sacrificing of the organism and lengthy procedures. The development of transgenic organisms has led to the establishment of simpler, more rapid, and less error-prone methods for detection of gene expression, often in a whole, living organism.

Transgenic organisms are generated by the introduction of foreign DNA into a host. Two types of transgenic organisms may be generated: transient (or mosaic) and stable. In the former, the foreign DNA element does not integrate with the host's DNA, and as a result transient transgenic organisms do not express foreign DNA in all cells and it may not be inherited by

future generations. In contrast, stable transgenic lines are generated after a recombination event between the introduced transgene and host DNA has occurred in germline cells. When organisms with the transgene present in their gametes are crossed, their progeny will contain the transgene in all cells, including the germline. These are stable lines as the transgene is passed from generation to generation. Transient systems are easier and take less time to generate, but have limitations. In a transiently transgenic organism only a few cell types or cell populations will contain the transgene; thus to identify all the cells or tissues impacted by a toxicant many hundreds to thousands of individuals must be screened and the expression pattern recorded. A consequence of such mosaic transgenic systems is that if insufficient numbers of individuals are screened some of the affected or target tissues and cells may be undetected or underestimated. In comparison, stable transgenic organisms have the transgene present in every cell, thus all cells or tissues expressing the transgene are observable in one individual. Generation of stable transgenic organisms is more time consuming but results in greater consistency between individual animals and experiments.

Transgenes for biomonitoring are constructed by fusing the promoter, hormone response element, or even an inducible gene of interest to a detectable reporter gene such as GFP, luciferase, or β-galactosidase. Several different transgenic organisms have been used for toxicological studies including zebrafish (Blechinger et al., 2002a; Seok et al., 2006), *Drosophila melanogaster* (e.g. Mukhopadhyay et al., 2003), *Caenorhabditis elegans* (Candido and Jones, 1996), and mice (Wirth et al., 2002; Operana et al., 2007), and these organisms have been used to assess the toxic effects of many different compounds and mixtures. For example, induction of *hsp70* expression in larvae of a transgenic strain of *Drosophila melanogaster* has been used to assess cellular toxicity of effluents (Mukhopadhyay et al., 2003), pesticides (Nazir et al., 2003;

Gupta et al., 2007), and municipal leachates (Bhargav et al., 2008). In these studies, expression of the transgene was also related to development of the larvae and reproductive performance as adults. Transgenic strains of *Caenorhabditis elegans*, with reporter genes for heat shock protein 16, have also been used for direct assessment of the toxicity of polluted river water (Mutwakil et al., 1997) and heavy metals (Chu and Chow, 2002). Additionally, transgenic zebrafish strains with reporter genes for *hsp70* expression or estrogen receptor activation have been utilized to assess toxicities of metals (Blechinger et al., 2002a; Seok et al., 2006) and estrogenic compounds (Bogers et al., 2006), respectively, in developing zebrafish. Also, zebrafish embryos transiently expressing GFP under control of the aryl hydrocarbon response element were exposed to dioxin and localized GFP expression was shown to be an accurate predictor of dioxin-induced gross developmental abnormalities (Mattingly et al., 2001).

Using transgenic organisms for toxicology studies is advantageous as cell- or tissue-specific reporter gene activation aids in identification of target sites in a whole organism and may lead to an understanding of how a compound causes an effect; however, transgenic organisms can be time-consuming to create and maintain. In comparison, transgenic cell lines may have advantages in this regard, as some lines are readily available, and are ideal for screening of many compounds in a short period. For example, the MELN cell line is a human breast cancer cell line that has a estrogen-regulated luciferase gene stably transfected; this line has recently been used to screen endocrine disrupting chemicals (Berckmans, et al., 2007) and for evaluation of similar compounds in drinking water supplies (Cargouet et al., 2007). Human cell lines have also been used in evaluation of metals (Tully et al., 2000) and industrial wastes (Ait-Aissa et al., 2003). A main disadvantage of cell line systems is that meaningful extrapolations to whole organisms are difficult to make, and as a result these tests are often done

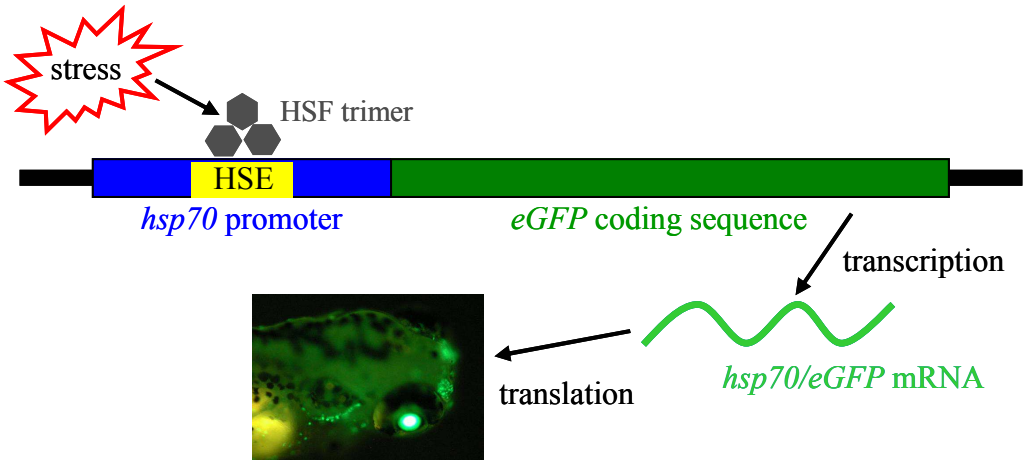
in conjunction with whole organism tests (Ait-Aissa et al., 2003; Cargouet et al., 2007) to increase the predictive power of the analysis. Additionally, cell lines are of less value in developmental studies where whole organism approaches are required.

1.7.1 *Hsp70/eGFP* transgenic zebrafish reporter system

Much work regarding heat shock proteins in the developing zebrafish has been accomplished in the laboratory of Dr. Patrick Krone, including the cloning and characterization of *hsp47*, *hsp70*, and *hsp90* expression during normal development and after environmental stress (reviewed in Krone et al., 1997; Krone et al., 2003). One member of the *hsp70* gene family, *hsp70-4*, was found to have little expression at control temperatures, but was strongly induced after heat shock and slightly induced after ethanol exposure (Lele et al., 1997). The promoter region of the *hsp70-4* gene was cloned and used to create a line of stable transgenic zebrafish (Halloran et al., 2000). For this reporter system, the *hsp70* promoter was linked to the coding sequence for enhanced green fluorescent protein (eGFP) to create the *hsp70/eGFP* transgene construct. The *hsp70* promoter drives expression of eGFP specifically in cells that activate the heat shock response pathway. As this promoter contains the HSE regulatory element, the eGFP coding region is transcribed following stress-induced HSF1 trimerization (see Figure 1.2). Once the *hsp70/eGFP* mRNA is translated, cells expressing the transgene are readily identified in whole, living zebrafish larvae using fluorescence microscopy due to the presence of eGFP in the stressed cell.

The stable line of transgenic zebrafish was generated by micro-injecting wild-type zebrafish embryos with the transgene construct (Halloran et al., 2000). These embryos were reared to maturity and pairwise crosses were done to identify any fish that passed the transgene

Figure 1.2 Expression of the *hsp70/eGFP* transgenic reporter system. Stress conditions in a cell activate the heat shock response pathway. HSF trimers bind to the *hsp70* promoter of the transgene and activate expression of the gene, resulting in eGFP accumulation in the cells which are readily identified via fluorescence microscopy.


stress
HSF trimer
HSE
hsp70 promoter
eGFP coding sequence
transcription
hsp70/eGFP mRNA
translation

to their progeny. Transgenic progeny are only observed if at least one parent had the transgene present in their gametes; that is a recombination event has occurred to put the transgene into the genome of germline cells. To identify which parent had passed on the transgene, parents were crossed to wild-types and these embryos assessed for the transgene. Transgenic progeny from this cross were reared to maturity, crossed with wild-type zebrafish, and their progeny were assessed for the transgene. Of these progeny, any transgenic embryos were assumed to have the transgene stably present in their genome, as it had been passed on for two generations and the progeny appeared biologically normal. From these first stably transgenic zebrafish, the line has been maintained for many generations.

Initial investigations with this transgenic line of zebrafish found expression of the transgene closely mimicked expression of endogenous *hsp70* under stress and non-stress conditions (Halloran et al., 2000; Blechinger et al., 2002b), making it an ideal candidate as a reporter system. Additionally, under non-stress conditions, expression of the transgene is observable in the lens of the developing zebrafish, which is highly advantageous for rapidly identifying transgenic embryos or larvae.

For toxicological studies, the *hsp70/eGFP* transgenic zebrafish have proven to be a reliable indicator of cell-specific and dose-dependent cadmium (Blechinger et al., 2002a) and arsenic (Salisbury, 2006) exposures. This stable transgenic line offers many advantages for toxicity testing. First, because the line is stable each cell has the potential to express the eGFP reporter protein. Also, eGFP is nontoxic and relatively stable, whereas *hsp70* transcripts are highly transient after heat shock (Lele et al., 1997). This allows for detection of exposure even a few days after the toxicant is removed. Additionally, unlike β-galactosidase or luciferase reporter systems, eGFP expression can be detected using fluorescence microscopy without

addition of exogenous substrates and sacrificing of the organism. Test subjects can be maintained to observe effects throughout an exposure period and long after the exposure period has ended. Furthermore, identification of cells and tissue-specific expression of the transgene is quick and easy compared to other methods for detecting gene expression, such as in situ hybridization or immunohistochemistry.

1.8 Research objectives

The central objective of this project was study the effect of sublethal cadmium exposures on zebrafish larvae and in particular, the effects of these exposures on the olfactory system at cellular and functional levels. The specific objectives of this research project were as follows:

a) Quantification of whole body cadmium accumulation by larval zebrafish following sublethal exposures to assess concentration- and exposure length-dependency using graphite furnace atomic absorption spectroscopy (GFAAS).
b) Assessment of cadmium-induced heat shock stress response in the olfactory epithelium using transgenic *hsp70/eGFP* larval zebrafish.
c) Measurement of the cellular effects of sublethal cadmium exposure on the olfactory epithelium of larval zebrafish via assays for cellular death, histopathology, and scanning electron microscopy (SEM).
d) Functional measurement of the effects of sublethal cadmium exposures on the olfactory system of larval zebrafish using a behavioural assay.
e) Measurement of the impact of additional calcium on the toxicity of sublethal cadmium exposures in larval zebrafish.

f) Quantification of whole body cadmium accumulation by larval zebrafish after exposure to sublethal concentrations of cadmium with addition of calcium by inductively coupled plasma mass spectrometry (ICP-MS).

g) Assessment of the impact of additional calcium on cadmium-induced heat shock stress response in the olfactory epithelium using transgenic *hsp70/eGFP* larval zebrafish.

h) Measurement of the cellular effects of sublethal cadmium exposure with addition of calcium on the olfactory epithelium of larval zebrafish via assays for cellular death and histopathology.

i) Functional assessment of the impact of sublethal cadmium exposure with addition of calcium on the olfactory system of larval zebrafish using a behavioural assay.

Chapter 2

2. Materials and Methods

2.1 Zebrafish maintenance, breeding, and embryo care

2.1.1 Zebrafish maintenance

The standard procedures regarding zebrafish care were followed as outlined in *The Zebrafish Book* (Westerfield, 1995). Adult wild-type zebrafish were purchased from a local pet store and maintained in an Aquatic Habitats Flow-Through System (Apopka, FL) at room temperature in carbon filtered tap water, with a photoperiod of 14 h of light and 10 h of darkness. The line of stable transgenic *hsp70/eGFP* zebrafish was also maintained in this manner. This photoperiod keeps the adults in a reproductive state allowing for continual spawning. Adults were fed at least once a day with blood worms (Hikari, Hayward, CA), brine shrimp (Hikari), floating pellets (Aquatic Eco-Systems, Apopka, FL), or dry flake food (Nutrafin, Hagen, Montreal, QC).

2.1.2 Zebrafish husbandry

To obtain embryos, 5 to 7 adult fish were placed in a small 4.5 L (1 gallon) breeding tank lined with glass marbles on the bottom and kept at 28.5°C for optimal breeding. The fish spawn at the start of the photoperiod and the fertilized eggs sink to the bottom of the tank between the marbles to be collected. After spawning, adult zebrafish were returned to the larger maintenance tanks. Marbles were removed from the breeding tanks and the water was poured through a fine mesh net to collect the embryos. Breeding success varied substantially; normally 100 to 300 fertilized eggs were collected from each breeding tank.

2.1.3 Zebrafish embryo care

After collection, embryos were divided into groups of 50 to 100 and placed in sterile 25 mL plastic petri dishes with carbon-filtered tap water containing 0.05% methylene blue as a fungicide. Water was changed at least once a day and any debris or dead embryos were removed to prevent mold or bacterial growth. Embryos were maintained in an incubator at 28.5°C under the standard 14-hour light and 10-hour dark photoperiod. Embryos maintained under these conditions can be staged in hours post fertilization (hpf) according to established guidelines (Kimmel et al., 1995).

2.2 System water and exposure solutions

Average water quality for our system water was: ammonia, 0.296 as ppm N; pH 8.05; alkalinity, 83 mg/L as $CaCO_3$; and hardness, 132 mg/L as $CaCO_3$. All exposures were conducted in sterile, 100 mm plastic petri dishes. Cadmium chloride hemi-pentahydrate ($CdCl_2 \cdot 2.5\ H_2O$; CAS # 7790-78-5) was purchased from J.T. Baker Inc. (Phillipsburg, NJ). Cadmium stock solutions of 1 and 0.1 mM were prepared in triple distilled water. Calcium chloride dihydrate ($CaCl_2 \cdot 2\ H_2O$; CAS # 10035-04-8) was purchased from Sigma Chemical Company (St. Louis, MO). A 5 mM calcium solution was made fresh daily in carbon-filtered tap water. Treatment solutions were made fresh daily from dilutions of the cadmium stock in carbon-filtered tap water and/or calcium solution to a final volume of 25 mL.

2.3 Larval zebrafish exposures

2.3.1 Cadmium exposures

A range of sublethal cadmium concentrations (0.5, 1.0, 5.0, and 10.0 μM Cd) were purposely chosen below the previously reported LC_{50} (median lethal concentration) of 18.8 μM Cd for larval stage zebrafish over a 96 h exposure period (Blechinger et al., 2002a). For the exposures, 72 hpf zebrafish larvae were placed in plastic petri dishes and continuously exposed to cadmium for up to 96 h with daily solution changes. Following the exposure period(s), larvae were treated as required for various subsequent analyses as detailed below. For assessment of dose-response relationships, exposures were conducted as replicates of 20 or 25 larvae per petri dish. Observations of morphological effects were made daily; dead larvae were recorded and removed. For statistical analysis of mortality and non-lethal morphological effects, an arcsine transformation was applied to observed percent mortality or percent non-lethal effects. One-way analysis of variance (ANOVA) with Student-Newman-Keuls post-hoc tests were performed using GraphPad InStat version 3.05 (GraphPad Software, San Diego, CA).

2.3.2. Cadmium and calcium co-exposures

For cadmium and calcium co-exposures, a range of sublethal cadmium concentrations (1.0, 5.0, and 10 μM Cd) were chosen for the reasons outlined in section 2.3.1. For calcium co-treatment with these selected cadmium concentrations, 1 and 5 mM Ca were chosen as low and high calcium treatments, respectively. Zebrafish larvae (72 hpf) were placed into plastic petri dishes and continuously exposed to cadmium, calcium, or in combination for 96 h. For assessment of dose-response relationships, exposures were conducted as replicates of 25 larvae per petri dish. Observations of morphological effects were made daily; dead larvae were

recorded and removed. For statistical analysis of mortality and non-lethal morphological effects, an arcsine transformation was applied to observed percent mortality or percent non-lethal effects. Two-way ANOVA was performed using GraphPad Prism version 3.02 and post-tests to compare means were performed using GraphPad QuickCalcs Online Calculator for Scientists (<www.graphpad.com/quickcalcs/posttest1.cfm>).

2.4 Zebrafish larvae photography

For light microscopic observations, whole larvae were viewed on a Leica MZ6 (Leica Microsystem, Wetzlar, Germany) dissecting microscope. Images of whole larvae were captured with a Nikon Coolpix (Nikon, Tokyo, Japan) digital camera mounted in an eyepiece. Images were processed using Adobe Photoshop Elements (Adobe Systems Incorporated, San Jose, CA) and arranged in Microsoft PowerPoint (Microsoft, Redmond, WA).

2.5 *Hsp70/eGFP* transgenic zebrafish

2.5.1 Detection of *Hsp70/eGFP* expression and identification of transgenic larvae

Expression of eGFP was detected using fluorescence microscopy (further described in section 2.5.3). A green excitation emission filter for eGFP was used to view the transgenic larvae. Potential *hsp70/eGFP* larvae are screened prior to use for expression of the transgene in the lens. Constitutive lens expression of *hsp70* and *hsp70/eGFP* is part of normal lens development (Blechinger et al., 2002b; Evans et al., 2005), and is not due to any toxicant-induced stress. This constitutive expression allows for separation of transgene positive larvae from the pool of collected embryos. Once identified, transgenic larvae were utilized in experiments to detect cell- and tissue-specific *hsp70/eGFP* expression in whole, living larvae.

The original transgenic line was created in an inbred strain of zebrafish. To improve breeding success, these transgenic fish were out-crossed to wild-type zebrafish. These larvae were screened for constitutive expression of the reporter gene in the lens. Transgenic individuals were reared to maturity and used as breeding stock. Transgenic offspring derived from the out-crossed line have expression patterns and levels identical to the original transgenic line, under non-stress and stressed conditions.

2.5.2 Exposures with *hsp70/eGFP* transgenic zebrafish larvae

Transgenic larvae exposed continuously to sublethal concentrations of cadmium (as described in section 2.3.1; n = 25 per petri dish) were observed at 0, 4, 8, 12, 24, 48, 72, and 96 h of the exposure period for expression of the *hsp70/eGFP* reporter gene. For the cadmium and calcium co-exposures, transgenic larvae exposed continuously to cadmium, calcium, or in combination (as described in section 2.3.2; n = 25 per petri dish) were observed at 0, 24, 48, 72 and 96 h of the exposure period for expression of the *hsp70/eGFP* reporter gene. For both sets of exposures, tissues expressing the transgene were recorded and images of the whole larvae were taken at each time point for all treatments.

2.5.3 Transgenic zebrafish photography

Living embryos were placed in a depression slide and viewed on a Nikon Eclipse E600 photomicroscope. Fluorescence was detected using a Nikon Y-FL Epi-fluorescence attachment and eGFP filter (excitation wavelength 488 nm; emission wavelength 507 nm). Images were captured with a Nikon Coolpix digital camera mounted directly in the microscope. Images were processed and arranged as described in section 2.4.

2.6 Zebrafish larvae fixation

For TUNEL staining and embedding, following the exposure period groups of 25 larvae were fixed in 4% paraformaldehyde (PFA) in phosphate buffered saline (0.03 M PBS; 4.3 mM monosodium phosphate (NaH_2PO_4), 137 mM sodium chloride (NaCl), 2.7 mM potassium chloride (KCl), 1.4 mM monopotassium phosphate (KH_2PO_4)) in 1.5 mL microcentrifuge tubes. They remained in the fixative for 2 hours at room temperature or overnight at 4°C. Once fixed, larvae were washed twice in PBST (0.03 M PBS with 1% Tween-20) and then gradually dehydrated through a graded series of washes in PBST/methanol (25%: 75%, 50%: 50%, 25%: 75%); all washes were 5 min in length. After washing twice in 100% methanol, larvae were stored at -20^{o}C in 100% methanol. Before use in TUNEL assays or embedding stored larvae were gradually rehydrated via successive washes of increasing PBST to methanol concentration.

2.7 Detection of cell death using the terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling assay

2.7.1 Background

Detection of dying cells was achieved using the TUNEL assay (terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling). This system is a fast and precise technique designed to identify apoptotic cell death at the single-cell level in tissues. During apoptosis, DNase catalyzed degradation of DNA generates small, double-stranded DNA fragments and introduces single strand breaks into large DNA fragments (Bortner et al., 1995). In the TUNEL reaction, terminal deoxynucleotidyl transferase attaches fluorescently labeled nucleotides to the free 3′OH-ends of the nicked and cleaved DNA. Because many labeled nucleotides are incorporated into the nuclei of cells with fragmented DNA, these cells are easily identified when

viewed with a fluorescence microscope. However, reports in the literature indicate that nick end labeling techniques (ex. TUNEL) cannot discriminate between apoptosis and necrosis (Grasl-Kraupp et al., 1995; Charriaut-Marlangue and Ben-Ari, 1995); i.e. these techniques are non-specific assays for cell death. Therefore, this assay was used to identify dying cells, regardless of cell death pathway.

2.7.2 Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling assay

Rehydrated larvae were washed three times for 30 minutes in PBST. All washes were at room temperature with gentle agitation unless noted otherwise. The larvae were permeabilized with 20 μg/ml Proteinase K (Roche Diagnostic Canada, Laval, PQ) in 10 mM Tris-HCl (pH 7.5) for 20 minutes at room temperature followed by two washes for 5 minutes in PBST. The larvae were secondarily fixed in 4% PFA for 30 minutes. Paraformaldehyde was removed with two washes for 5 minutes in PBST. The larvae were incubated in freshly prepared TUNEL reaction buffer (200 mM sodium cacodylate, 1 mM cobalt chloride, 25 mM Tris-HCl, 2.5 mg/mL bovine serum albumin, pH 6.6) for 5 minutes at room temperature, followed by 1 hour at 4°C. The reaction buffer was removed and 50 μL of TUNEL reaction solution (45 μL TUNEL tetramethylrhodamine-dUTP Label and 5 μL TUNEL enzyme mixed immediately before use; Roche Diagnostic Canada, Laval, PQ) was added. The samples were incubated in the dark for 3 hours at 37°C. After the incubation, samples were washed twice for 5 minutes in PBST and post-fixed for 30 minutes in 4% PFA at room temperature in the dark. Two washes for 5 minutes in PBST were done to remove the PFA. The samples were washed overnight at 4°C in the dark with gentle agitation to remove any excess label. In the morning, the PBST was changed and the samples were viewed with fluorescence microscopy.

To assure the integrity of each TUNEL assay internal positive and negative controls were performed. For the positive control, one set of untreated larvae was incubated with 100 µg/mL DNase I (Amersham Biosciences, Piscataway, NJ) in Mg-free PCR buffer (New England Biolabs, Beverly, MA) for 1 hour at 37°C prior to addition of the TUNEL reaction solution. The DNase treatment introduces DNA nicks in nuclei throughout the larvae for the labeled nucleotides to be incorporated. For the negative control, the 5 µL of TUNEL enzyme was omitted from the reaction mixture. Samples were incubated in TUNEL label only and serve as a control for background fluorescence due to non-specific binding of labeled-dUTP.

2.7.3 Fluorescence microscopy and photography

Following completion of the TUNEL protocol, larvae were placed on a depression slide and viewed on a fluorescent photomicroscope as described in section 2.5.3. To view the red fluorescence of the TUNEL label, a tetramethylrhodamine isothiocyanate (TRITC) filter was utilized (excitation wavelength 547 nm; emission wavelength 572 nm). Images were captured, processed, and arranged as described in section 2.4.

2.8 Zebrafish larvae embedding and sectioning

2.8.1 Glycol methacrylate (JB-4) plastic embedding and sectioning

Rehydrated, fixed larvae to be embedded were oriented in molten, 1% agarose gel (w/v) using blunt probes. Once properly positioned, the agarose was allowed to cool and harden. When set, the gel was cut forming a small block containing the larvae; the gel block was placed in 100% ethanol to dehydrate with gentle agitation for 5-8 hours at 4°C. After dehydration, the blocks were processed for embedding as per manufacturer's instructions (PolySciences Inc,

Warrington, PA). Agarose blocks were infiltrated with JB-4 embedding Solution A (monomer) and benzoyl peroxide catalyst (100 mL solution A to 1.25 g of catalyst) at 4°C overnight, with gentle agitation. In the morning, infiltration solution was replaced to allow for complete removal of alcohol and tissue fluids. Infiltration was allowed to continue for another 6-8 hours. Completely infiltrated agarose blocks were set in plastic embedding molds, filled with JB-4 embedding solution (freshly prepared infiltration solution and JB-4 Solution B (accelerator); 25 mL infiltration solution: 1 mL Solution B), and covered with a metal chuck. Molds were placed at 4^{o}C overnight to polymerize. Hardened blocks were sectioned using a Sorvall Porter-Blum (Ivan Sorvall Inc, Norwalk, CT) microtome with a glass knife to 5.5 µm thickness. Sections were floated in a water bath and placed on glass slides. Slides were heated to evaporate any water and adhere the section to the glass. Plastic sections were used for methylene blue-azure II staining.

2.9 Zebrafish larvae histology

2.9.1 Methylene blue-azure II staining

JB-4 sections were stained with methylene blue-azure II stain, a non-differential basophilic stain, (13 µg/mL methylene blue, 2 µg/mL Azure II, 1% glycerol (v/v), 1% methanol (v/v), 1% phosphate buffer (0.1 mM NaH_2PO_4, 0.67 mM KH_2PO_4; v/v)) for 10 seconds at 65°C, and washed for 5 minutes in distilled water (Humphrey and Pittman, 1974). Stained slides were dried and coverslipped using a xylene based mounting material (Cytoseal XYL, Richard-Allen Scientific, Kalamazoo, MI).

2.9.2 Light microscopy and photography

Prepared slides were viewed using a Nikon Eclipse E600 photomicroscope and conventional white light source. Images were captured, processed, and arranged as described in section 2.4.

2.10 Scanning electron microscopy (SEM)

2.10.1 Sample preparation

Larvae used for electron microscopy were fixed in PFA (as described in section 2.7) and dehydrated with slight modification. After washes in PBST, embryos were dehydrated in a graded series of washes in PBST/acetone (80%:20%, 60%:40%, 40%:60%, 20%:80%); all washes were 10 minutes in length. Larvae were then washed twice in 100% acetone and stored at -20°C in 100% acetone. Dehydrated larvae were critical point dried with liquid carbon dioxide, mounted on aluminum stubs, and gold coated with an Edwards S150B (BOC Edwards, Crawley, UK) sputter-coater; these preparation steps were conducted by Dr. X. Wu (Biology Department, University of Saskatchewan).

2.10.2 Scanning electron microscopic analysis

Samples were examined using a Phillips 505 scanning electron microscope. SEM was conducted with the assistance of Dr. X. Wu (Biology Department, University of Saskatchewan). Images were captured with a Polaroid (Waltham, MA) camera. Photographs were scanned to create digital images. These images were processed and arranged as described in section in section 2.4.

2.11 Quantification of cadmium accumulation in larval zebrafish

2.11.1 Graphite furnace atomic absorption spectroscopy (GFAAS)

2.11.1.1 Background

Atomic absorption spectroscopy (AAS) is a widely used method to determine the concentration of a single element in analytical samples. This technique is based on the theory that metal atoms in the gas phase will absorb ultraviolet light at characteristic frequencies. A cadmium-specific hollow cathode lamp is used as the light source and a graphite furnace atomizes the samples. Cadmium atoms in the sample absorb incoming light at 228.8 nm and a change in light intensity is measured by the detector. Working from a standard curve, the cadmium concentration in samples is determined.

2.11.1.2 Sample preparation

Larvae were exposed to cadmium for 24, 48, 72, or 96 h (30 larvae per petri dish) as described in section 2.3.1. After the exposure period, larvae were washed five times in carbon-filtered system water. Ten larvae per replicate were placed in pre-weighed glass vials. Any water was removed using a micro-pipettor and the vial reweighed to determine the amount of fish tissue in each sample. Five hundred µL of 69% HNO_3 (OmniPure, Aldrich, St. Louis, MO) was added to each and the samples were left at room temperature overnight to completely digest the tissue and solubilize any cadmium in the sample. After digestion, 500 µL of triple-distilled water was added to each sample for a final volume of 1 mL. Three replicates were prepared for each cadmium exposure concentration and exposure length. Samples were analyzed immediately or stored at 4^{o}C for up to a week.

2.11.1.3 Graphite furnace atomic absorption spectrometry sample analysis

Analysis was performed with a Varian SpectraAA 220Z (Varian Instruments, Palo Alto, CA). Six µL of sample and 6 µL 2% $NH_4H_2PO_4$ (m/v; Baker Analyzed A.C.S. Reagent, J.T. Baker) were dispensed into a platform graphite tube at 85°C. Ammonium phosphate monobasic ($NH_4H_2PO_4$) is a matrix modifier utilized in cadmium analysis to allow for higher ashing temperatures. Operating conditions were: 12 s at 95°C and 15 s at 120°C to dry the sample; ashing at 15 s at 500°C to remove organics; and, atomization at 2000°C for 3 s. Argon gas was bled through the system to sweep drying and ashing products; gas flow was stopped during atomization and absorption. The absorbance of radiation from the hollow cathode lamp at 228.8 nm by cadmium atoms was measured and background corrected using the Zeeman method. A standard curve was generated from cadmium standards ranging from 0 to 1.50 /L Cd. Using the measured cadmium content in each sample and weight of the sample, the amount of cadmium accumulation by zebrafish larvae per wet weight (as ng/g) was determined for each sample.

2.11.2 Inductively coupled plasma mass spectrometry (ICP-MS)

2.11.2.1 Background

Inductively coupled plasma mass spectrometry (ICP-MS) is becoming increasingly popular for elemental analysis due to its low detection limits and good accuracy and precision. An ICP torch is used to generate high temperatures to atomize and ionize elements in injected samples. These elemental ions are identified and quantified by a mass spectrometer.

2.11.2.2 Sample preparation

Larvae were exposed to cadmium, calcium, or in combination for 96 h (50 larvae per petri dish) as described above in section 2.3.2. Following the exposure period, the larvae were

rinsed several times in fresh system water. All water was removed passing the larvae over an acid-washed fine mesh. About 100 larvae for each sample replicate were placed between two pieces of plastic. The larvae were enclosed between the pieces of plastic by heat sealing the edges, forming little plastic bags, which were stored at -20°C until further sample preparation and analysis. For each exposure group, three replicate samples (~100 larvae per sample) were prepared. Final sample preparation was performed by Prairie Diagnostic Services (Saskatoon, SK). For final sample preparation, the fish samples were weighed and digested in a MARS-Express microwave (CEM Corporation, Matthews, NC) with 0.5 mL of concentrated HNO_3 for 15 min at 180°C. After digestion 50 µL of a 1 ppm yttrium and indium internal standard was added. The sample was made up to a 5 mL final volume with double distilled water for analysis.

2.11.2.3 Inductively coupled plasma mass spectrometry sample analysis

Prepared samples were analyzed for cadmium content with an ICP-MS X-Series (Thermo Jarrell-Ash Corporation, Waltham, MA) by Prairie Diagnostic Services. The quantified cadmium content in the sample and weight of the sample were used to calculate cadmium accumulation by the zebrafish larvae (as ng/g wet weight). For statistical analysis of cadmium accumulation by larval zebrafish, Two-way ANOVA was performed using GraphPad Prism version 3.02 and post-tests to compare means were performed using GraphPad QuickCalcs Online Calculator for Scientists (<www.graphpad.com/quickcalcs/posttest1.cfm>).

2.12 Zebrafish behavioural assay to test olfactory function

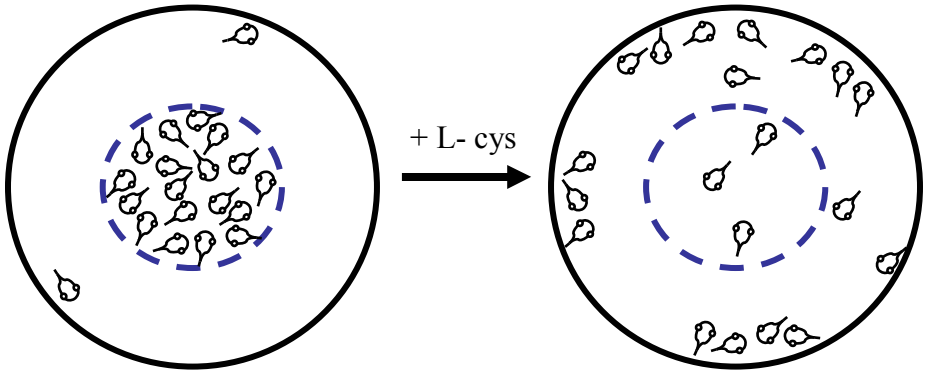
2.12.1 Larval zebrafish aversion to L-cysteine

Olfactory function in larval zebrafish was assayed by measuring the aversion response to L-cysteine (L-cys), using a procedure described by Vitebsky et al. (2005) with modifications. Larvae were exposed to cadmium, calcium, or in combination for 96 h (25-50 larvae per petri dish) as described above in sections 2.3.1 and 2.3.2. Following the 96 h exposure period, larvae were transferred to fresh system water (i.e. no cadmium or additional calcium) and assessed for motility via contact stimulus. Non-motile and non-responsive larvae were removed and not included in behavior testing. For the assay, a 100 mm petri dish was placed on a white background centered on a 35 mm diameter circle, referred to as the inner circle. Larvae (~15 per trial) were transferred into the center of the petri dish containing 25 mL of system water, and 50 µL of an odorant or control stimulus was pipetted into the center with minimal disturbance. A schematic diagram of the assay is given in Figure 2.1. The odorant stimulus utilized was 200 mM L-cysteine (Sigma Aldrich), an odorant known to elicit an aversion response. The control stimulus utilized was distilled water, as it does not induce an aversion response, but represents any spontaneous response by zebrafish larvae. The proportion of fish that had completely exited the inner circle was recorded after 2 minutes.

For statistical analysis, an arcsine transformation was applied to the proportion of fish responding to the added stimulus (i.e. exited the inner circle) for each trial. For analysis of the effects of sublethal cadmium exposures on the olfactory system of larval zebrafish, one-way ANOVA with Student-Newman-Keuls post-hoc tests to compare all groups was performed using GraphPad InStat version 3.05. For analysis of olfactory function in larvae cadmium, calcium, and in combination, two-way ANOVA was performed using GraphPad Prism version 3.02 and

post-tests to compare means were performed using GraphPad QuickCalcs Online Calculator for Scientists (<www.graphpad.com/quickcalcs/posttest1.cfm>).

Figure 2.1 Schematic of L-cysteine aversion assay for olfactory-dependent behaviour. Larvae are located within the center circle (i.e. inner circle) prior to addition of the stimulus. After addition of L-cysteine stimulus, larvae exhibiting an aversion response swim to the outer edges of the petri dish. The proportion of fish that exited the inner circle was recorded for each trial, indicating the proportion of larvae eliciting an aversion response to L-cysteine.



Before stimulus

After 2 minutes

Chapter 3

3. Results

3.1 Sublethal cadmium exposures with zebrafish larvae

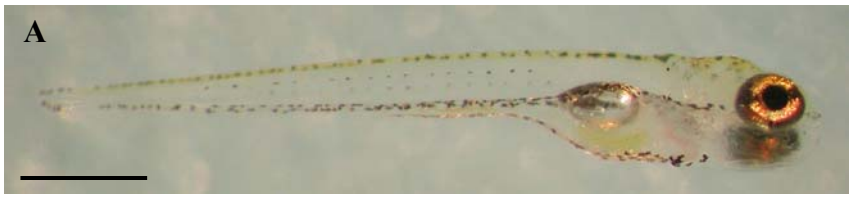
3.1.1 General observations of mortality and non-lethal morphological effects

To study the effects of cadmium on larval zebrafish, a range of sublethal exposure concentrations (0.5, 1, 5, and 10 μM Cd) was chosen that lie below the LC_{50} of 18.8 μM Cd and near the EC_{50} (median combined adverse effect concentration) of 1.7 μM Cd previously reported for larval zebrafish over a 96 h exposure period (Blechinger et al., 2002a). The exposures were termed "sublethal" as each cadmium concentration is less than the LC_{50}. Additionally, a range of concentrations allowed for assessment of dose-dependency in the observed effects at all levels investigated, from observations of the whole organism to cellular and molecular effects.

Gross effects of larvae exposed to cadmium included mortality, edema, and trunk abnormalities. Representative images of the non-lethal morphological effects are shown in Figure 3.1. Edema was usually observed in the thorax region (Figure 3.1, panel B), but also occurred in the head and pericardial regions. Trunk abnormalities or abnormal curvatures of the vertebrae included lordosis typified by dorsal concave curvature of the vertebral column (Figure 3.1, panel C), and kyphosis typified by dorsal convex curvature of the vertebral column (Figure 3.1, panel D). Incidences of mortality and these non-lethal morphological effects increased with cadmium exposure concentration (Figure 3.2, panels A and B). As expected from the known LC_{50}, the survival rate over the 96 h exposure period was greater than 75% in all treatment groups (Figure 3.2, panel A). Also, relative to control larvae, significant incidences of mortality were only observed in larvae exposed to the higher cadmium concentrations (i.e. 5 and 10 μM

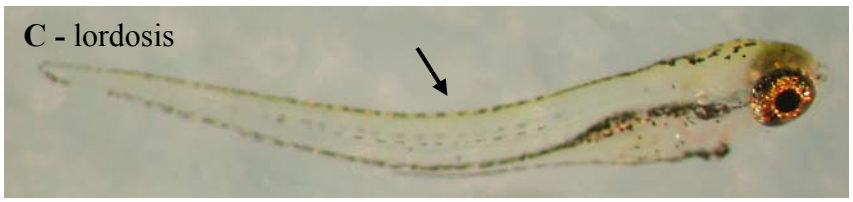
Figure 3.1 Non-lethal morphological effects caused by cadmium exposure. Zebrafish larvae exposed to sublethal concentrations of cadmium for 96 h had an increased incidence of edema (panel B) and trunk abnormalities (panels C and D), relative to control larvae (panel A). Larvae shown in panels B-D were exposed to 5 μM Cd for 96 h. Scale bar represents 0.5 mm.

No effects (Control)



Edema

Trunk abnormalities



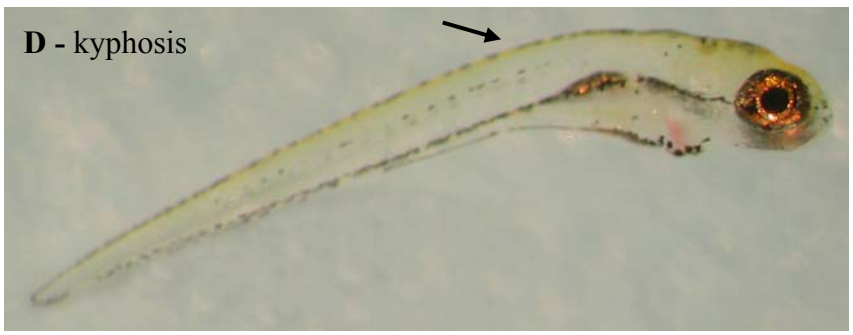
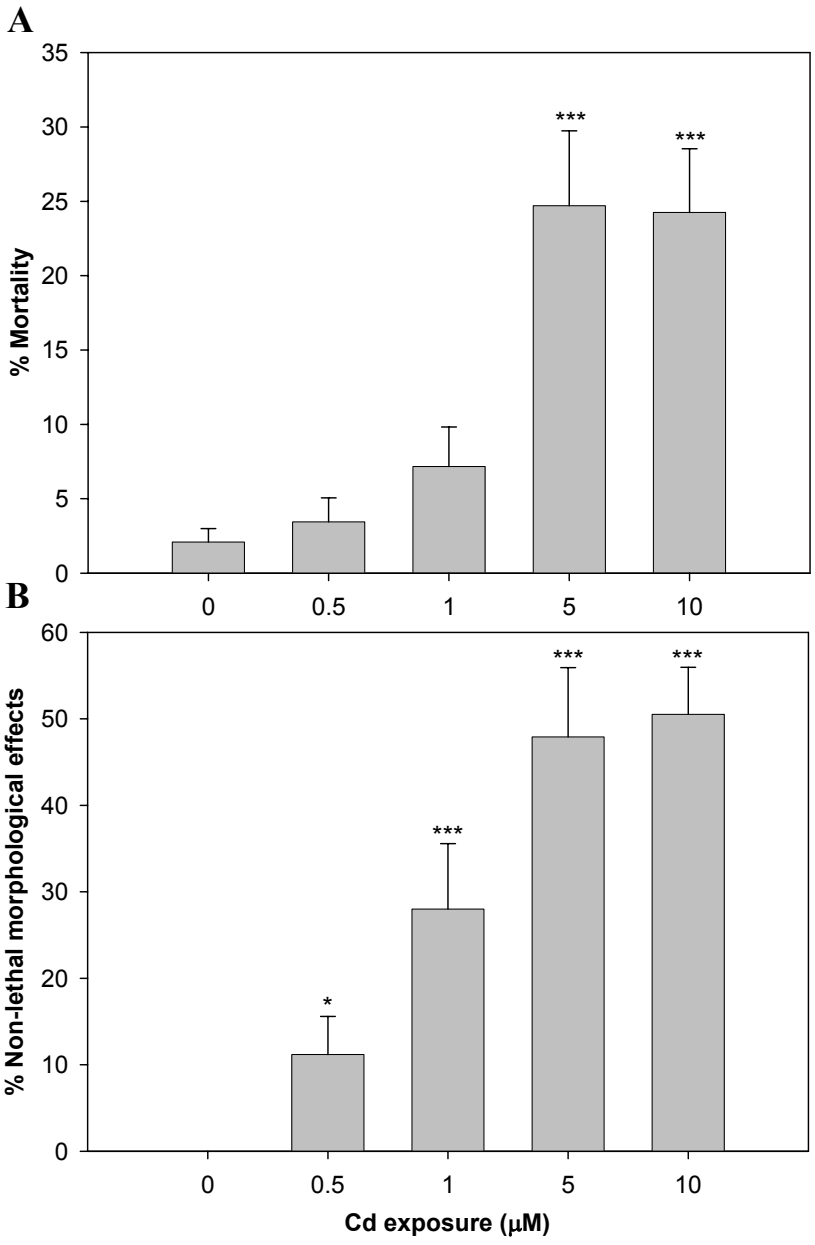


Figure 3.2 Dose-response relationships for 96 h sublethal cadmium exposures with larval zebrafish. Incidences (measured as percent) of mortality (Panel A) and non-lethal morphological effects (Panel B) increased with cadmium exposure concentration. Data represents an average of 8-12 replicates (20-25 larvae per replicate) and error bars represent standard error. One-way ANOVA with Student-Newman-Keuls post-hoc tests were used to compare between groups. * $p < 0.05$; *** $p < 0.001$

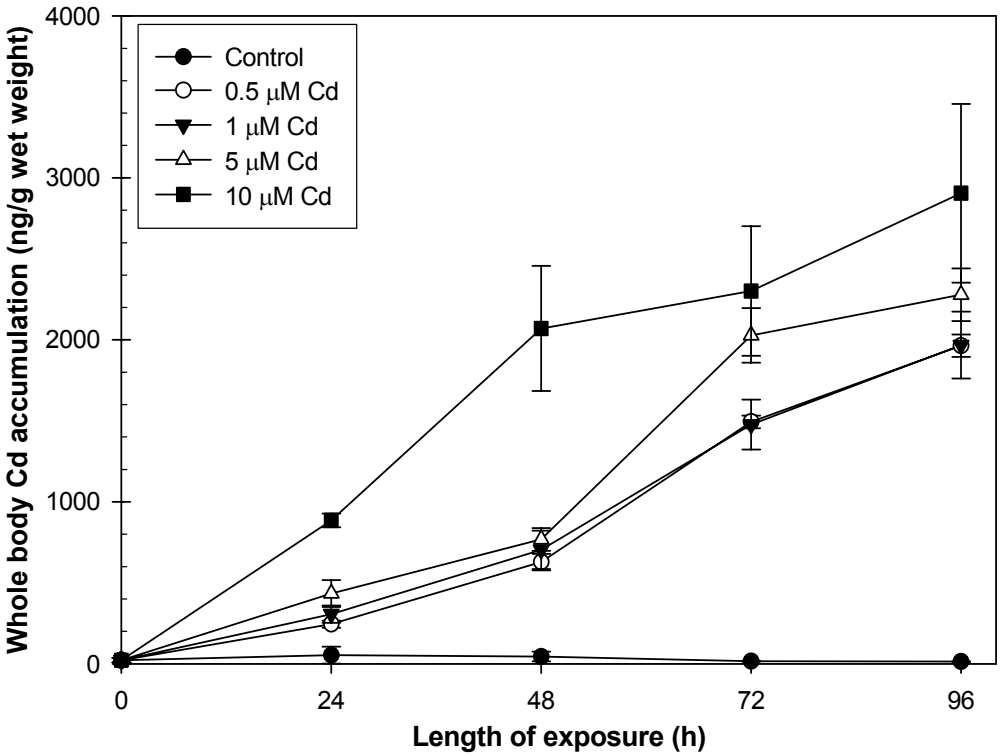

A
% Mortality
35
30
25
20
15
10
5
0
***
***
0
0.5
1
5
10
B
% Non-lethal morphological effects
60
50
40
30
20
10
0
*
***
***
***
0
0.5
1
5
10
Cd exposure (μM)

Cd; $p < 0.001$). In comparison, all cadmium exposure concentrations resulted in significant incidences of non-lethal morphological effects (Figure 3.2, panel B). For example, larvae exposed to the lowest cadmium concentration of 0.5 μM Cd had a significant incidence of non-lethal morphological effects ($p < 0.05$), as on average greater than 10% of larvae in this group developed edema or trunk abnormalities (Figure 3.2).

3.1.2 Time course of whole-body accumulation of cadmium by larval zebrafish using graphite furnace atomic absorption spectroscopy (GFAAS)

To generate a time course of cadmium accumulation in larval zebrafish at each exposure concentration, larvae were exposed to cadmium for 24, 28, 72, and 96 h and analyzed for cadmium content using GFAAS (Figure 3.3). Whole-body cadmium accumulation increased with both the length of exposure and exposure concentration. Larvae exposed to 10 μM Cd had the greatest amount of cadmium accumulation. In comparison, the lower cadmium exposure groups (0.5, 1, and 5 μM) had nearly identical uptake for the first 48 h of exposure despite a 10 fold difference in exposure concentration. Over the 96 h exposure period, larvae continued to accumulate cadmium and body burdens did not appear to level off in any of the cadmium exposure groups.

Figure 3.3 Time course of whole-body cadmium accumulation by larval zebrafish. Larvae (72 hpf) were continuously exposed to a range of sublethal cadmium concentrations. Samples were collected every 24 h over the 96 h exposure period and analyzed for cadmium content using GFAAS**.** Data represents an average of three replicates (10 larvae per replicate) and error bars represent standard error.


Whole body Cd accumulation (ng/g wet weight)
4000
3000
2000
1000
0
Control
0.5 μM Cd
1 μM Cd
5 μM Cd
10 μM Cd
0
24
48
72
96
Length of exposure (h)

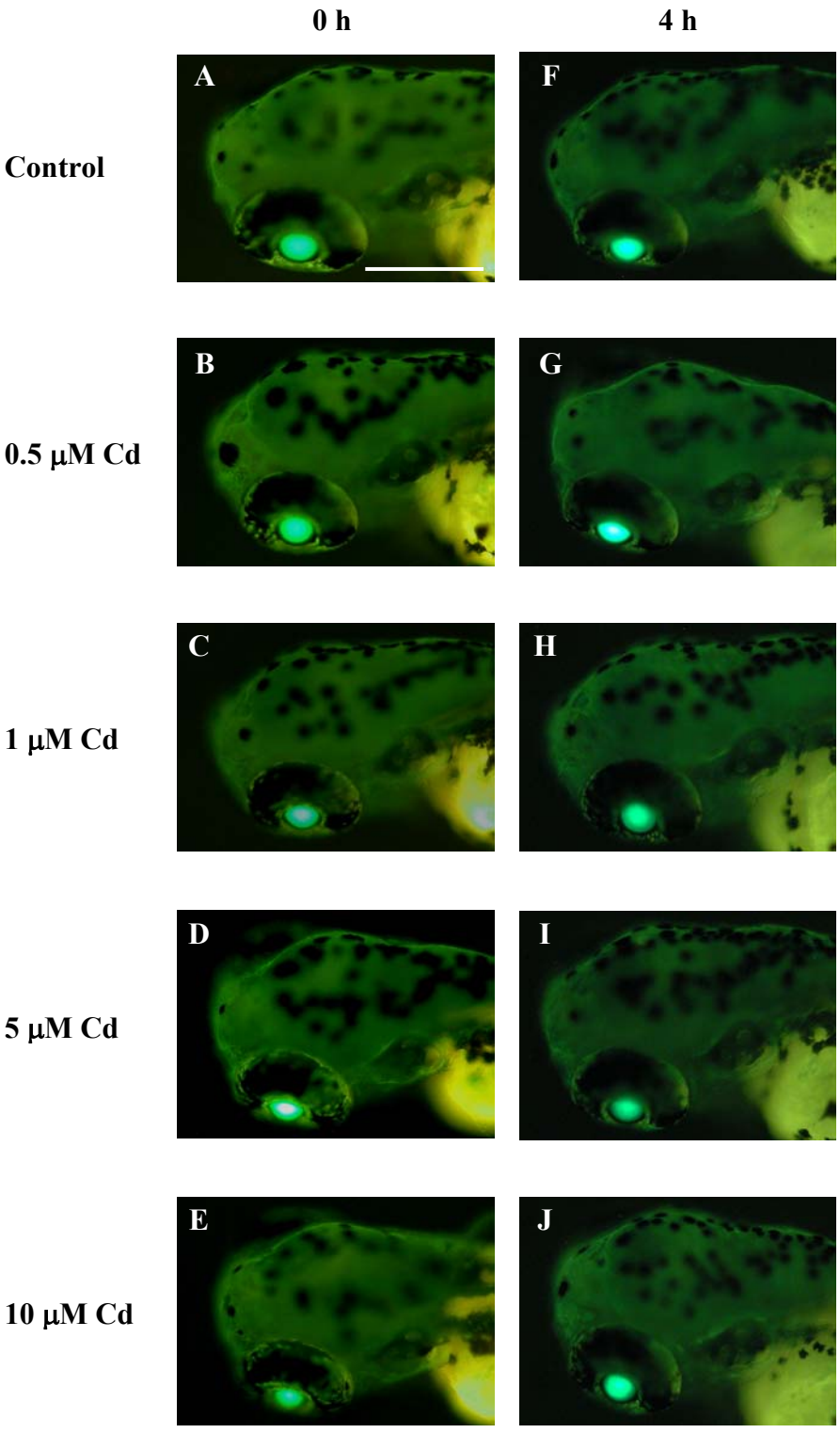
3.1.3 Time course of concentration-dependent expression of *hsp70/eGFP* over the 96 h exposure period

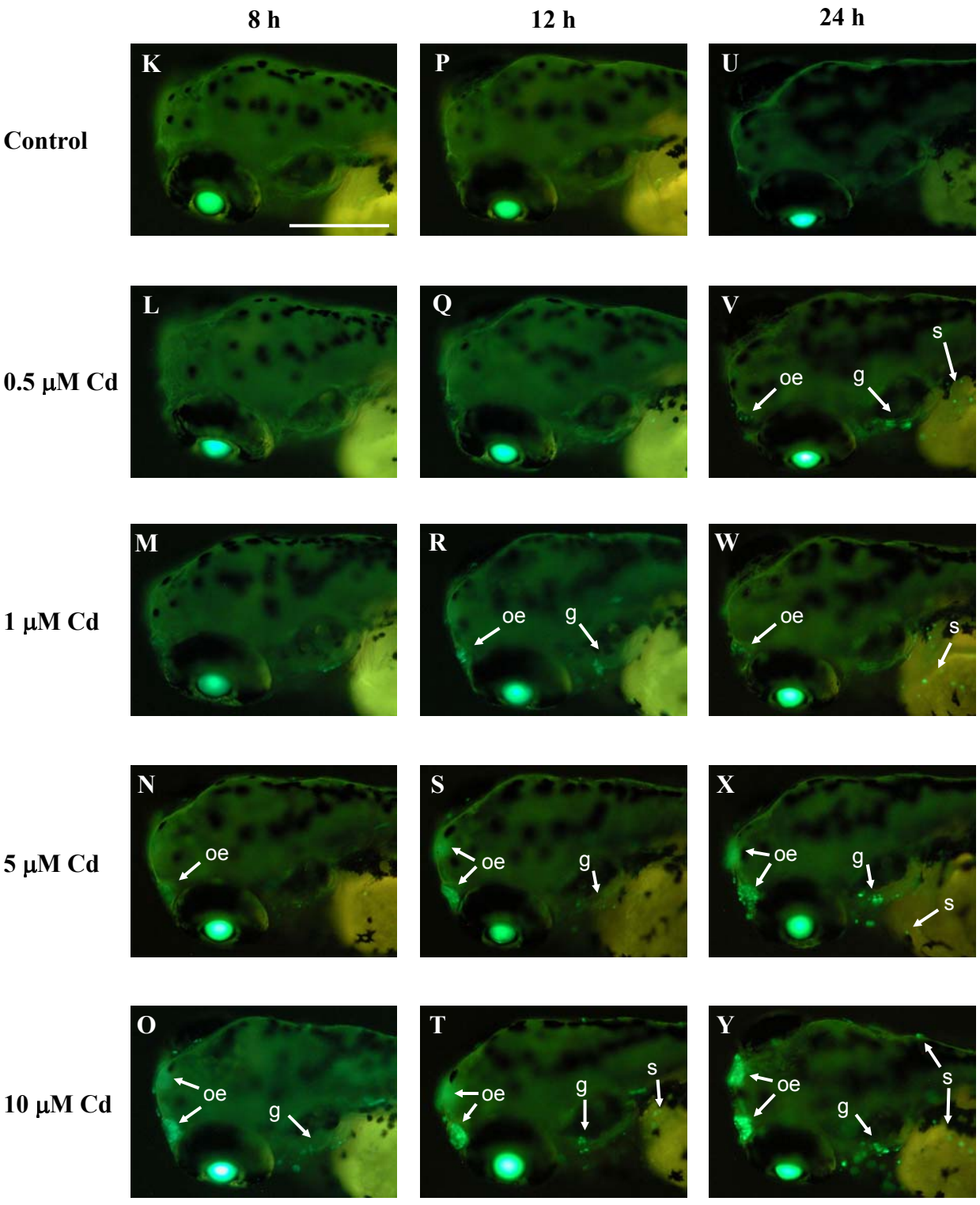
Transgenic *hsp70/eGFP* zebrafish larvae were utilized to initially characterize the effects of sublethal cadmium exposure at the cellular level. As shown in Figure 3.4, expression of the reporter construct (seen as eGFP fluorescence) was both dose- and time-dependent with expression occurring in cells of the gills, skin and olfactory epithelium. Larvae shown in Figure 3.4 are representative fish, as expression of the reporter gene in the olfactory epithelium, gills, and skin was consistent in every larva examined for each treatment group at every observation point. As expected from previous work, lens-specific expression of the transgene was evident in all fish, including control groups, due to constitutive lens expression of *hsp70* as a part of normal eye development (Halloran et al., 2000; Blechinger et al., 2002b). At all time points and concentrations, eGFP fluorescence was not observed in any control larvae other than this expected signal in the lens.

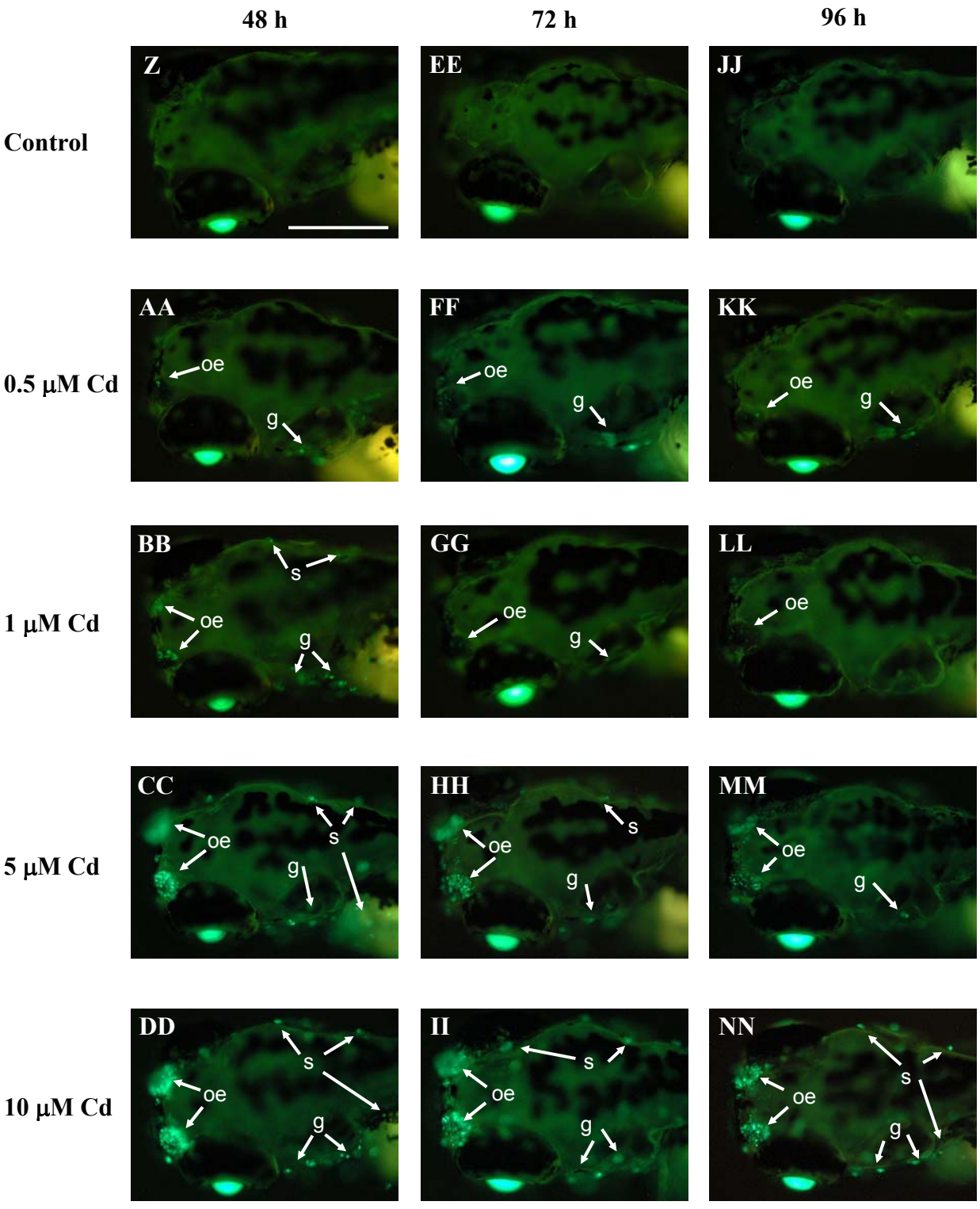
Given the known accumulation of cadmium in the olfactory system of fish (Tjalve et al., 1986) and cadmium-induced sensory impairment (Baker and Montgomery, 2001; Scott et al., 2003), expression patterns of *hsp70/eGFP* in the olfactory epithelium of zebrafish larvae were of particular interest. At the lower cadmium exposure concentrations of 0.5 and 1 μM, relatively weak fluorescence and a lower number of expressing cells were observed in the olfactory epithelium. The onset of eGFP fluorescence was first observed after 12 (Figure 3.4, panel R) and 24 h (Figure 3.4, panel V) of continuous cadmium exposure, for 1 and 0.5 μM Cd, respectively. In contrast, exposure at the higher concentrations of 5 and 10 μM Cd resulted in an earlier onset of transgene activation and a greater number of cells in the olfactory epithelium

Figure 3.4 Time course of concentration-dependent *hsp70/eGFP* expression in transgenic zebrafish larvae during a continuous cadmium exposure. Whole, living larvae were observed for expression of the *hsp70/eGFP* transgene every 4 h for the first 12 h and every 24 h for the remainder of the 96 h exposure period. Cadmium exposure induced concentration-dependent expression of the reporter gene (eGFP fluorescence) in the gills, olfactory epithelium, and skin. Onset of *hsp70/eGFP* expression in the olfactory epithelium was first observed at 8 h for 5 and 10 µM Cd (panels N and O), at 12 h for 1 µM Cd (panel R), and at 24 for 0.5 µM Cd. Strongest hsp70/eGFP fluorescence was observed at 48 h for all cadmium exposure groups (panels AA-DD), and was noticeably weaker at 72 h (panels FF-II) and 96 h (panels KK-NN) of the continuous exposure period. Scale bars represent 250 µm. g – gill, oe – olfactory epithelium, s – skin

Note: Lens expression of the reporter construct, observable in every larva, was due to constitutive *hsp70* expression as a part of normal lens development (Halloran et al., 2000; Blechinger et al., 2002b).


0 h
4 h
Control
0.5 µM Cd
1 µM Cd
5 µM Cd
10 µM Cd
A
B
C
D
E
F
G
H
I
J


8 h
12 h
24 h
Control
0.5 µM Cd
1 µM Cd
5 µM Cd
10 µM Cd
K
P
U
L
Q
V
M
R
W
N
S
X
O
T
Y
oe
g
s

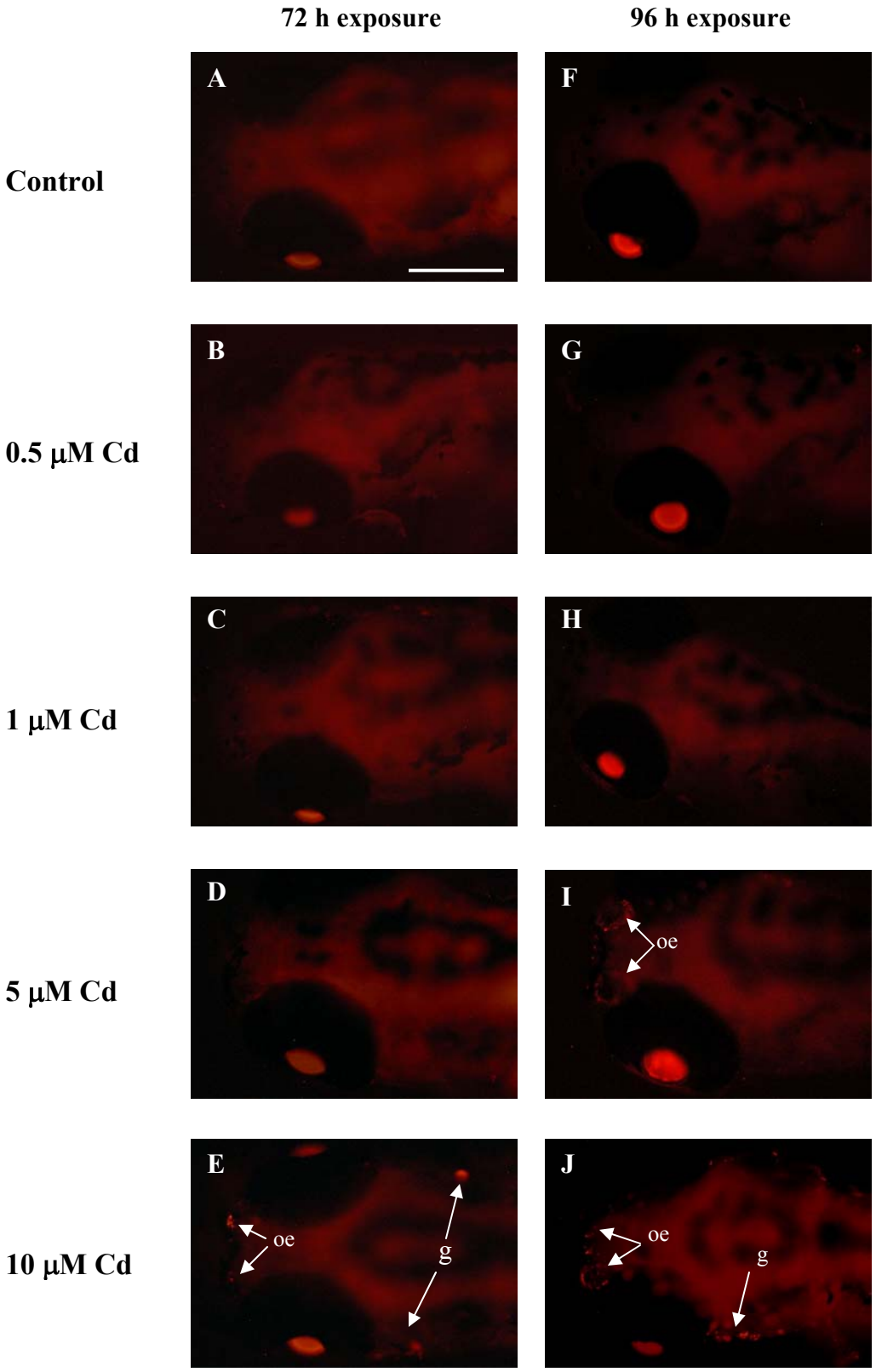

48 h
72 h
96 h
Control
0.5 µM Cd
1 µM Cd
5 µM Cd
10 µM Cd
Z
EE
JJ
AA
FF
KK
BB
GG
LL
CC
HH
MM
DD
II
NN
oe
g
s

expressing the reporter gene. Expression of *hsp70/eGFP* was first observed in the olfactory epithelium after 8 h of cadmium exposure at these higher concentrations (Figure 3.4, panels N and O). Additionally, high levels of eGFP fluorescence were observed in the olfactory epithelium of larvae exposed to 5 and 10 µM Cd at the 24 and 48 h time points (Figure 3.4, panels X, Y, CC, and DD). The circular organization of the external olfactory organ is clearly evident in these larvae. This relatively strong fluorescence was due to a large number of cells expressing *hsp70/eGFP* and expression of this reporter gene at relatively high levels, indicating a strong stress response in this tissue. Interestingly, eGFP fluorescence in the olfactory epithelium appeared to decrease at the 72 and 96 h time points, even though cadmium exposures were continuous through these observation times. This apparent loss of fluorescence strength was most evident at the higher exposure concentrations of 5 and 10 µM Cd, as noticeably fewer cells in the olfactory epithelium were expressing the reporter gene (Figure 3.4, panels HH, II, MM, and NN).

3.1.4 Cadmium-induced cell death in the olfactory epithelium

With the tissue-specific stress response in the olfactory epithelium of zebrafish larvae following sublethal cadmium exposure and previous reports of cadmium impairing olfactory function in fish (Baker and Montgomery, 2001; Scott et al., 2003), the potential for cell death in this tissue was investigated. Furthermore, I hypothesized that the decrease in *hsp70/eGFP* expression in the olfactory epithelium was likely be the result of either recovery of the cells from the chemical stress or death of the affected cells. To address this issue, cell death (TUNEL) assays were performed on larvae following 72 and 96 h sublethal cadmium exposures (Figure

Figure 3.5 Cadmium-induced cell death in zebrafish larvae following 72 and 96 h exposures measured using fluorescent TUNEL assays. TUNEL-positive cells (arrows) were observable in the olfactory epithelium and gill tissue of larvae exposed to 10 µM Cd for 72 (panel E) and 96 h (panel J), and in the olfactory epithelium of larvae exposed to 5 µM Cd for 96 h (panel I). No cell death was observed, after either exposure period, in untreated control fish (panels A and F), nor in fish exposed to 0.5 µM Cd (panels B and G) and 1 µM Cd (panels C and H). Scale bar represents 250 µm. g – gill, oe – olfactory epithelium

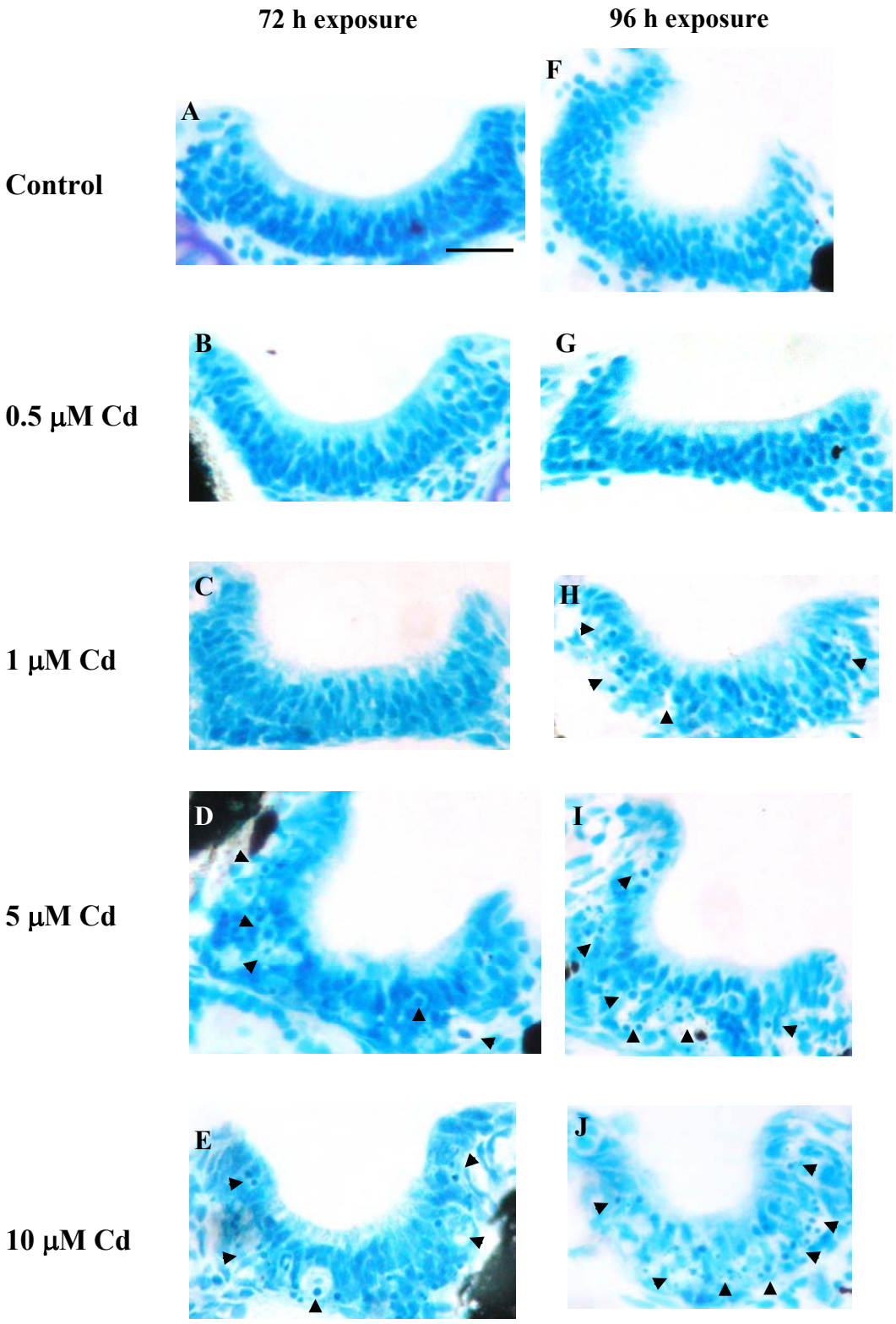

72 h exposure
96 h exposure
Control
0.5 µM Cd
1 µM Cd
5 µM Cd
10 µM Cd
A
B
C
D
E
F
G
H
I
J
oe
g
oe
g
oe
g

3.5), as both these exposure periods correspond to the observed decreases in eGFP fluorescence in transgenic larvae. After a 72 h cadmium exposure, a small number TUNEL-positive, dying cells were observed in the olfactory epithelium and gills of larvae from the highest exposure group of 10 μM Cd (Figure 3.5, panel E). No cell death was detected in larvae from the lower cadmium exposure groups at this time point (Figure 3.5, panels B-D). Following a 96 h exposure period, numerous dying cells were observed in the olfactory epithelium of larvae from both the 5 and 10 μM Cd exposure groups (Figure 3.5, panels I and J). The circular organization and anatomical location of the TUNEL-labeling at this exposure time point clearly corresponds to the olfactory pits expressing *hsp70/eGFP* in the transgenic larvae. Similar to the shorter exposure period, no cell death was observed in larvae exposed to the lower cadmium concentrations of 0.5 and 1 μM (Figure 3.5, panels G and H) for 96 h.

3.1.5 Histological effects of cadmium exposure on the olfactory epithelium

The observed decrease in eGFP fluorescence and TUNEL-labeling suggested that changes were occurring in the olfactory epithelium after 72 and 96 h continuous cadmium exposures; therefore, histological analysis of this tissue was performed at these time points (Figure 3.6). In contrast to the densely packed cells in the olfactory tissue from control larvae, irregular clear areas of cell loss and less densely arranged cells were observed in the olfactory epithelium of larvae exposed to 5 and 10 μM Cd for 72 h (Figure 3.6, panels D and E) and 1, 5, and 10 μM Cd for 96 h (Figure 3.6, panels H, I, and J). Often apparent in the areas of cell loss were small, round, darkly stained structures. These structures were not observed in any sections of unexposed control larvae olfactory tissue, and most likely represent cellular debris. Histopathological effects were again found to be time- and dose-dependent. The extent of cell

Figure 3.6 Histopathology of zebrafish larvae olfactory epithelium following 72 and 96 h sublethal cadmium exposures. Abnormal histopathology was observed in 5 (panel D) and 10 (panel E) μM Cd exposure groups following 72 h exposure. Additionally, histological alterations were evident after 96 h exposure to 1 (panel H), 5 (panel I), and 10 (panel J) μM Cd. Histopathological alterations included less dense arrangement of cells relative to control tissue (panels A and F) and irregular areas of cell loss (arrowheads) often associated with small, round, darkly stained structures in these areas. No histological effects were observed in the 0.5 μM Cd exposure group (panels B and G). Scale bar represents 20 μm.


72 h exposure
96 h exposure
Control
0.5 µM Cd
1 µM Cd
5 µM Cd
10 µM Cd
A
B
C
D
E
F
G
H
I
J

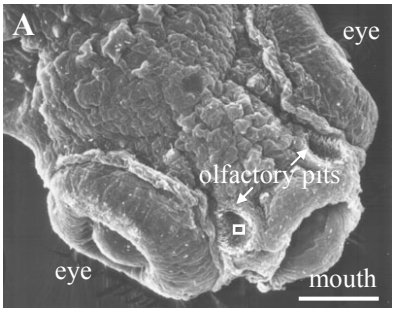
loss and tissue alterations was evident at the higher sublethal cadmium exposures of 5 and 10 μM and most obvious after a 96 h exposure period (Figure 3.6, panels I and J). No effects were observed in the olfactory epithelium of larvae exposed to 1 μM Cd for 72 h (Figure 3.6, panel C). However, after a 96 h exposure this exposure group represented a transition between control tissue and severe effects observed at the higher cadmium exposures. The abnormal histopathology was less severe in the larvae exposed to 1 μM Cd for 96 h with less extensive areas of cellular loss and fewer small, round, darkly stained structures (Figure 3.6, panel H). Histological analyses of the olfactory epithelium of larvae exposed to 0.5 μM Cd were similar to control, as no obvious alterations were observed at either exposure length (Figure 3.6, panels B and G).

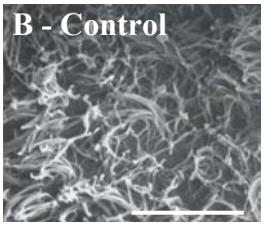
3.1.6 Topographical analysis of olfactory epithelium following cadmium exposure

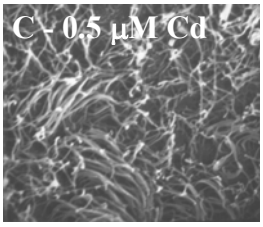
To further characterize the effects of sublethal cadmium exposures on the olfactory epithelium of zebrafish larvae, the surface topography of this tissue was imaged using scanning electron microscopy (SEM). In zebrafish larvae, the pair of olfactory pits is situated above the mouth and anterior to the eyes (Figure 3.7, panel A) and the olfactory epithelium lining the pits is exposed to the external environment. The sensory cilia located in the centre of the olfactory epithelium were readily visualized using the high magnification capabilities of SEM and analyzed for cadmium-induced effects following a 72 h exposure (72 h SEM results are preliminary; Figure 3.7). In unexposed, control larvae, numerous sensory cilia were observed across the central portion of the olfactory epithelium (Figure 3.7, panel B). Thick mats of sensory cilia were also observed in larvae from the lower cadmium exposure groups of 0.5 and 1 μM (Figure 3.7, panels C and D). In contrast, 72 h exposures to 5 and 10 μM Cd resulted in

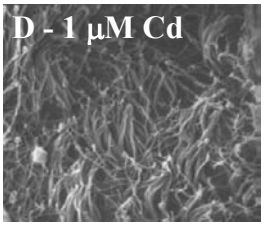
Figure 3.7 Topographical analysis of the olfactory epithelium of zebrafish larvae following 72 h sublethal cadmium exposure.
Panel A: The olfactory pits are located above the mouth and anterior to the eyes on the head of larval zebrafish. Inset box represents the area of olfactory epithelium shown in subsequent panels. Scale bar represents 100 μm.

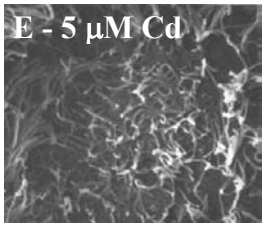
Panels B-I: Sensory cilia were discernible as long thread-like structures in the central region of the olfactory pits using SEM. Numerous cilia were observed on the olfactory epithelium of unexposed control larvae (B), and larvae exposed to 0.5 (C) and 1 (D) μM Cd. Following exposure to 5 (E) and 10 (F) μM Cd noticeably less sensory cilia were present. The olfactory pit appeared shrunken or collapsed following exposure to 10 μM Cd (F). Scale bar represents 10 μm.

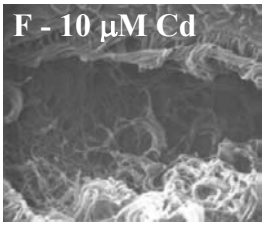

A
eye
olfactory pits
eye
mouth


B - Control


C - 0.5 μM Cd


D - 1 μM Cd

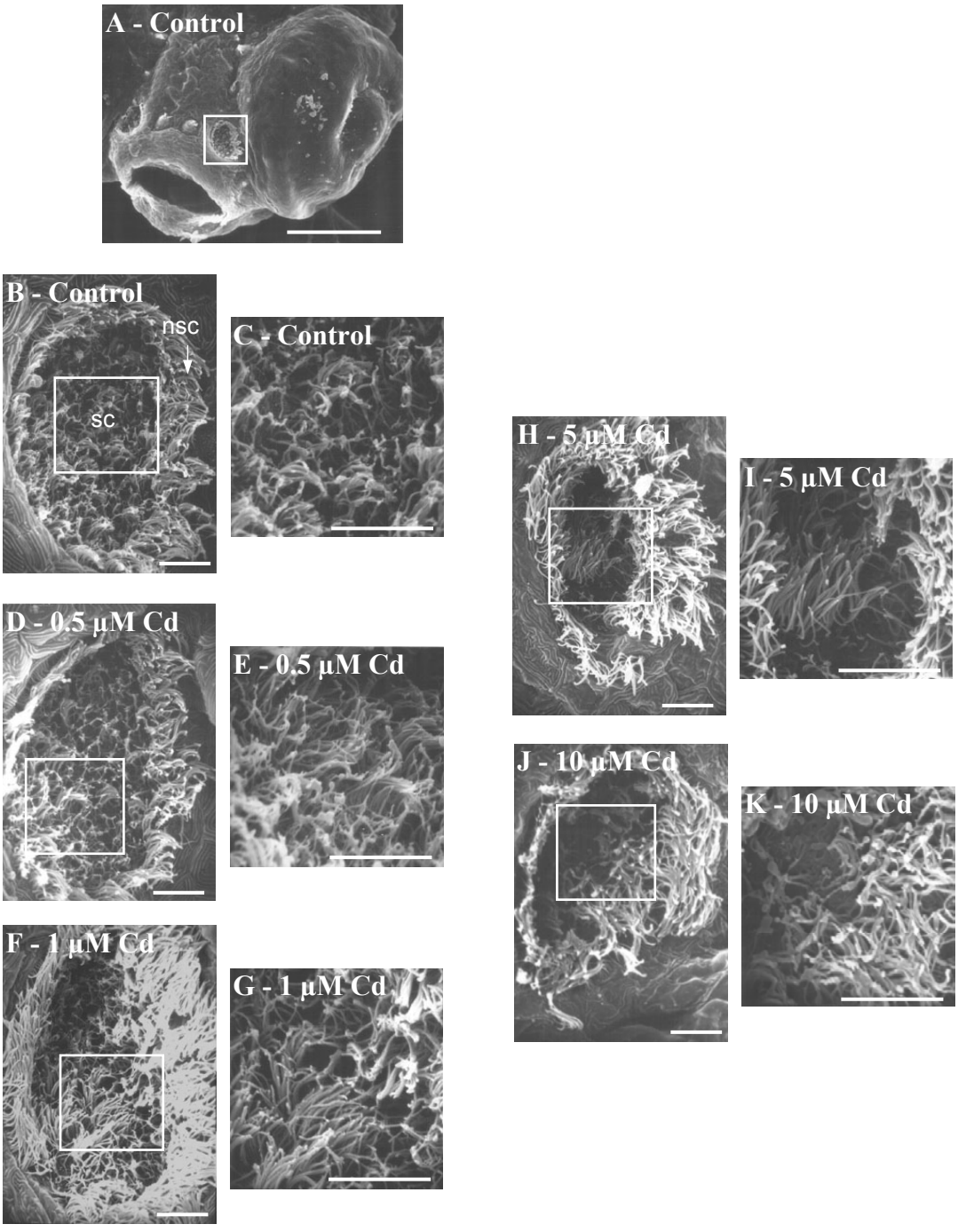

E - 5 μM Cd


F - 10 μM Cd

changes to the surface of the olfactory epithelium (Figure 3.7, panels E and F). Fewer sensory cilia in the central portion of the olfactory epithelium were observed following exposure at these higher cadmium concentrations. Also, following a 72 h exposure to 10 μM Cd areas of the olfactory epithelium lacked sensory cilia and the olfactory pit appeared shrunken or collapsed (Figure 3.7, panel F).

Topographical analysis of the olfactory epithelium was also performed following a 96 h exposure period (Figure 3.8). Similar to the results following a 72 h sublethal cadmium exposure, changes in the topography of the olfactory epithelium were observed in larvae exposed to 5 and 10 μM Cd (Figure 3.8, panels H-K). More specifically, in these larvae, large areas of the floor of the olfactory pit containing few sensory cilia were interspersed with areas containing cilia. Additionally, the entire olfactory pit appeared to be collapsed and misshapen in larvae exposed to the higher cadmium concentrations (Figure 3.8, panels H and J). In contrast, the entire floor of the olfactory pit in control embryos was covered with a thick mat of sensory cilia (Figure 3.8, panels B and C). Exposure to 0.5 and 1 μM Cd for 96 h had no discernable effects on surface appearance of the olfactory epithelium, as many sensory cilia were present throughout the olfactory pits (Figure 3.8, panels D-G). Interestingly, the long, non-sensory kinocilia were unaffected in all treatment groups forming a fringe around the outer edge of the olfactory pit in all larvae examined.

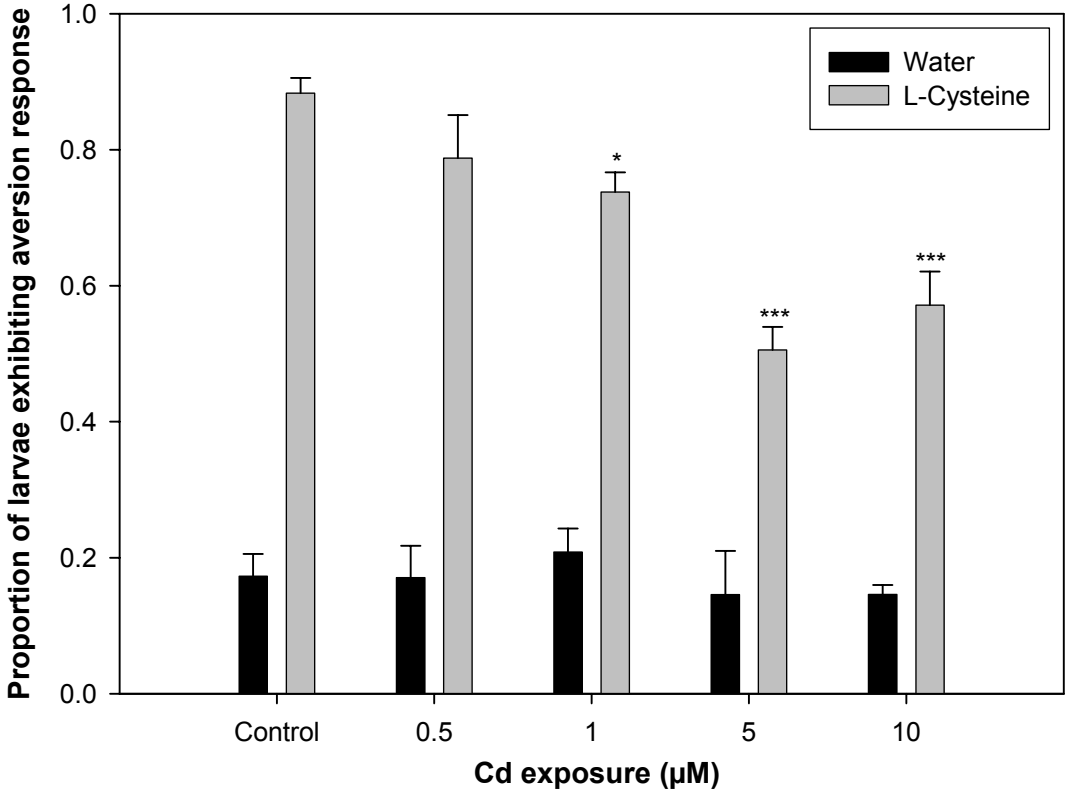
Figure 3.8 Surface topography of zebrafish larvae olfactory epithelium following 96 h sublethal cadmium exposure. Exposure to 5 (panels H and I) and 10 (panels J and K) μM Cd clearly impacted the ciliated sensory cells. Fewer cilia were observed and large regions were devoid of sensory cilia. In comparison, the surface of the olfactory epithelium of larvae exposed to 0.5 (panels D and E) and 1 (panels F and G) μM Cd was similar to control (panels B and C) with numerous sensory cilia present throughout the centre of the olfactory pit. The long, non-sensory kinocilia located around the outer edge of the olfactory pit were unaffected by cadmium exposure. Inset boxes indicate area shown in following panel. Scale bars represent: A – 100 μm, B-K – 10 μm. nsc – non-sensory ciliated cells, sc – sensory ciliated cells


A - Control
B - Control
nsc
sc
C - Control
D - 0.5 µM Cd
E - 0.5 µM Cd
F - 1 µM Cd
G - 1 µM Cd
H - 5 µM Cd
I - 5 µM Cd
J - 10 µM Cd
K - 10 µM Cd

3.1.7 Behavioural analysis of zebrafish larvae following 96 h cadmium exposure

With the observed alterations to the olfactory epithelium described above (sections 3.1.4-3.1.6), the potential for sublethal cadmium exposure to impact functional aspects of the olfactory sensory system was investigated. At the end of the 96 h exposure period, larvae were tested for an olfactory-based aversion response to L-cysteine to examine olfactory function (assay modified from Vitebsky et al., 2005). Typically, unexposed control larvae displayed a rapid response to the L-cysteine stimulus as they quickly swam to the outer edges of the petri dish, exiting the inner circle within seconds of stimulus addition, and remained at the perimeter for the two minute duration of the assay. In comparison, cadmium exposed larvae often: i) had a slower response time to the L-cysteine stimulus; ii) would swim away from the centre of the petri dish, where the stimulus was added, but not exiting the inner circle; iii) displayed an initial aversion to the stimulus, but would re-enter and remain in the inner circle before the end of the assay period. The impact of 96 h sublethal cadmium exposures was found to be dose-dependent (Figure 3.9). The average proportion of larvae exposed to 1, 5, and 10 μM Cd that responded to the L-cysteine stimulus was significantly decreased compared to the average proportion of unexposed control larvae responding to the same stimulus ($p < 0.05$ for 1 μM Cd; $p < 0.001$ for 5 and 10 μM Cd). Larvae exposed to 5 μM Cd had the lowest average response to L-cysteine; however the difference in average proportion responses was not significantly different between the 5 and 10 μM Cd exposure groups. Additionally, although the average proportion response to L-cysteine for larvae in the lowest exposure group of 0.5 μM Cd was lower than observed with control larvae, the difference was not found to be significant. No statistically significant differences were found between responses to the distilled water control stimulus between the control and cadmium exposure groups.

Figure 3.9 Behavioural response of zebrafish larvae to L-cysteine following 96 h sublethal cadmium exposures. Impact of cadmium exposure on the olfactory system was assessed by measuring the aversion response to an L-cysteine stimulus, relative to a distilled water stimulus (see Figure 2.1 for schematic diagram of the assay). Exposure to cadmium resulted in a decreased response to the L-cysteine stimulus, indicating that olfactory function had been impaired. Data shown represents average of at least 6 trials (~15 larvae/trial). Error bars represent standard error. One-way ANOVA with Student-Newman-Keuls post-hoc tests were performed to compare between groups. * $p < 0.05$, *** $p < 0.001$


Proportion of larvae exhibiting aversion response
1.0
0.8
0.6
0.4
0.2
0.0
Water
L-Cysteine
*
***
***
Control
0.5
1
5
10
Cd exposure (μM)

3.2 Sublethal cadmium and calcium co-exposures with zebrafish larvae

3.2.1 General observations of mortality and non-lethal morphological effects

To study the potential for calcium to ameliorate the effects of cadmium exposures on larval zebrafish, larvae were exposed to a range of sublethal cadmium concentrations with a high or low calcium co-treatment. The sublethal cadmium concentrations of 1, 5 and 10 μM Cd were chosen for these investigations. These are the same cadmium concentrations utilized for the studies described in section 3.1, with the lowest cadmium exposure of 0.5 μM omitted. This exposure level was omitted as in almost all the investigations described above larvae in this group were similar to control group larvae and any ameliorating effect of calcium would be difficult to identify at this cadmium concentration. For the calcium co-exposures, 1 and 5 mM Ca were chosen as high and low calcium treatments as they represented large excesses of cadmium. Additionally, 2 mM Ca for several days does not affect the survival of developing zebrafish (Meinelt et al., 2001). Furthermore, using two concentrations of calcium co-treatment allowed for assessment of dose-dependency of amelioration of adverse effects at a given cadmium exposure level.

Incidences of mortality and abnormal morphological endpoints in larvae from the 1 and 5 mM Ca treatment groups (i.e. no cadmium) were not significantly different from untreated control larvae (i.e. no cadmium or calcium; Figure 3.10, panels A and B). In larvae exposed to cadmium, a dose-dependent pattern of increasing mortality and non-lethal morphological effects with increasing cadmium concentration was observed, regardless of calcium treatment. The non-lethal morphological effects observed were mainly edema and trunk abnormalities, as previously described in section 3.1.1. However, when zebrafish larvae were co-exposed to cadmium and excess calcium, the incidence of mortality and these non-lethal morphological effects decreased

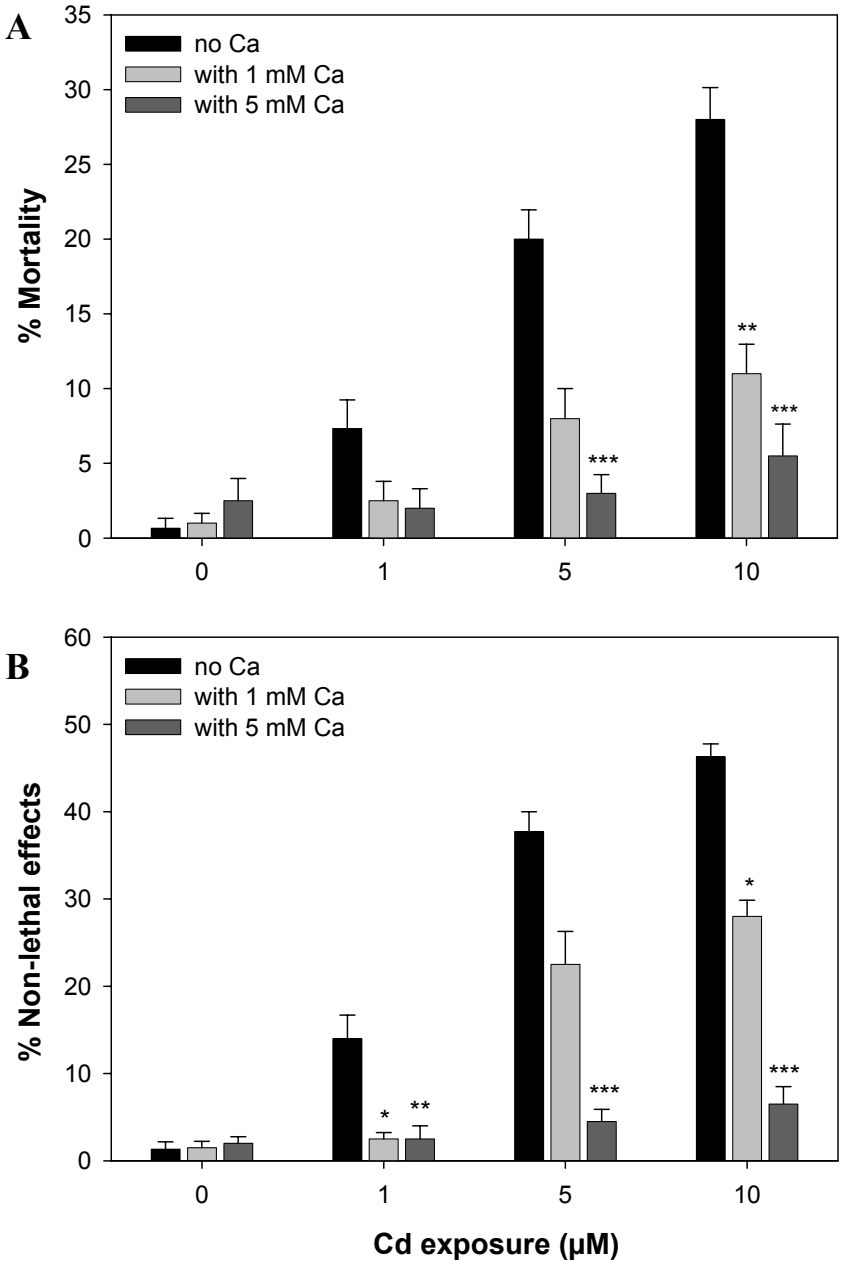
Figure 3.10 Dose-response relationships for larval zebrafish exposed to cadmium, calcium, or in combination for 96 h. Incidences (measured as percent) of mortality (Panel A) and non-lethal morphological effects (Panel B) increased with cadmium exposure concentration and decreased with calcium co-treatment concentration. Data represents an average of 6-8 replicates (25 larvae per replicate) and error bars represent standard error. Two-way ANOVA with post-tests were used to analyze the effects of cadmium and calcium on mortality or non-lethal morphological effects in zebrafish larvae. Summaries of two-way ANOVA analyses are given below (Mortality – Table 3.1; Non-lethal morphological effects – Table 3.2).
* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$

Table 3.1 Two-way ANOVA p-values for mortality in zebrafish larvae exposed to cadmium, calcium, or in combination

Source of variation	p-value
Cadmium	<0.0001
Calcium	<0.0001
Interaction	0.0048

Table 3.2 Two-way ANOVA p-values for non-lethal morphological effects in zebrafish larvae exposed to cadmium, calcium, or in combination

Source of variation	p-value
Cadmium	<0.0001
Calcium	<0.0001
Interaction	<0.0001


A
no Ca
with 1 mM Ca
with 5 mM Ca
% Mortality
35
30
25
20
15
10
5
0
**
***
***
0
1
5
10
B
no Ca
with 1 mM Ca
with 5 mM Ca
% Non-lethal effects
60
50
40
30
20
10
0
*
**
*
***
***
0
1
5
10
Cd exposure (μM)

(Figure 3.10, panels A and B), relative to cadmium exposure alone. The interaction between cadmium and calcium was significant for mortality ($p<0.0048$) and non-lethal morphological effects ($p<0.001$). Furthermore, the incidence of these endpoints was decreased to a greater extent at the high calcium co-treatment relative to the low calcium co-treatment. Co-exposure to 1 mM Ca significantly reduced mortality in the 10 μM Cd exposure group ($p < 0.01$) compared to cadmium exposure alone (Figure 3.10 panel A). In comparison, a decrease in mortality was observed with 5 and 10 μM Cd when co-exposed to 5 mM Ca ($p < 0.001$), but not at the 1 or 5 μM Cd exposure levels. Additionally, co-treatment with 5 mM Ca significantly lowered the occurrence of edema and trunk abnormalities at all cadmium concentrations ($p < 0.01$ for 1 μM Cd; $p < 0.001$ for 5 and 10 μM Cd; Figure 3.10, panel B). In comparison, the incidence of morphological effects in the 1 and 10 μM Cd was significantly reduced when the larvae were co-exposed to 1 mM Ca ($p < 0.05$).

3.2.2 Whole-body accumulation of cadmium in the presence of additional calcium by larval zebrafish using inductively coupled plasma-mass spectrometry (ICP-MS)

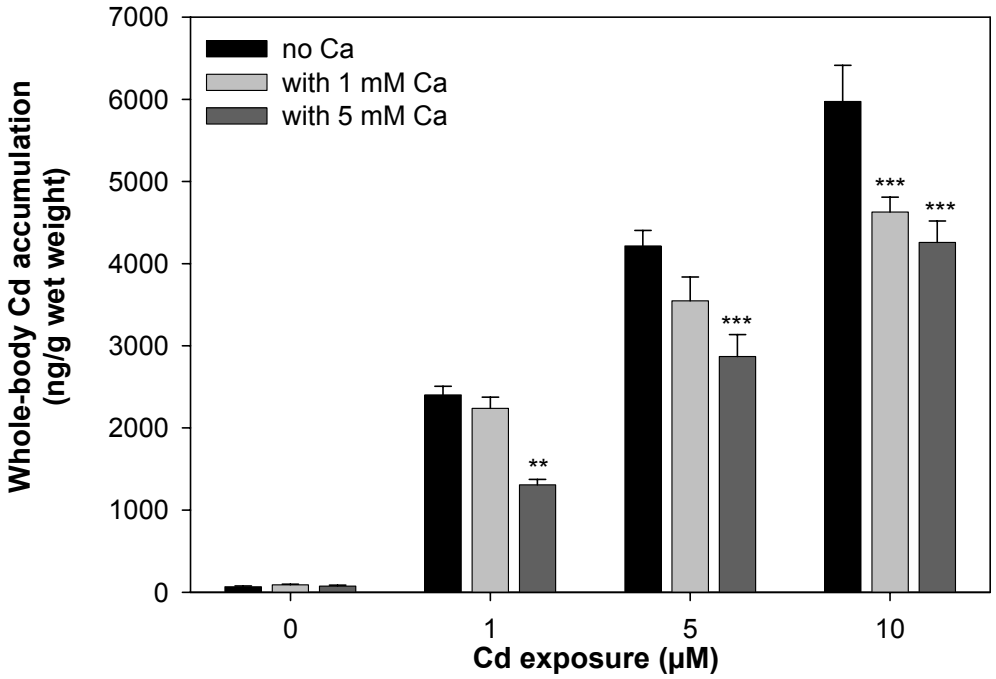
To determine if calcium co-treatments were limiting cadmium uptake, larvae were analyzed for whole-body cadmium accumulation following the 96 h exposure period by ICP-MS. The GFAAS utilized for cadmium quantification in section 3.1.2 was not operational at the time of this analysis necessitating a change in analytical techniques to ICP-MS.

As expected, whole-body cadmium accumulation in zebrafish larvae increased with cadmium exposure concentration (Figure 3.11). When zebrafish larvae were co-exposed to cadmium and calcium, the amount of cadmium accumulated decreased ($p<0.0053$ for the

Figure 3.11 Effect of calcium co-treatment on whole-body cadmium accumulation in larval zebrafish following a 96 h exposure period. Whole-body cadmium accumulation was quantified by ICP-MS. Cadmium accumulation increased with cadmium exposure concentration. Co-treatment with 1 or 5 mM Ca decreased cadmium accumulation with increasing calcium co-treatment. At each cadmium exposure concentration, co-treatment with 5 mM calcium resulted in a significant decrease in whole-body cadmium accumulation. Three replicates (~100 larvae per replicate) were used for cadmium quantification by ICP-MS. Error bars represent standard error. Two-way ANOVA with post-tests were used to analyze the effects of calcium on cadmium uptake in zebrafish larvae. Summary of two-way ANOVA analysis is given below (Table 3.3).
** $p < 0.01$; *** $p < 0.001$

Table 3.3 Two-way ANOVA p-values for cadmium accumulation in zebrafish larvae exposed to cadmium, calcium, or in combination

Source of variation	p-value
Cadmium	<0.0001
Calcium	<0.0001
Interaction	0.0053


7000
6000
5000
4000
3000
2000
1000
0
no Ca
with 1 mM Ca
with 5 mM Ca
Whole-body Cd accumulation
(ng/g wet weight)
**
***
***
***
0
1
5
10
Cd exposure (μM)

interaction between cadmium and calcium). Furthermore, this observed decrease in cadmium accumulation was greatest with a high calcium co-exposure compared to the low calcium co-treatment (Figure 3.11). More specifically, the 1 mM calcium co-treatment reduced cadmium accumulation by 7%, 16%, and 23% in larvae exposed to 1, 5, and 10 μM Cd respectively, while the high calcium co-treatment decreased cadmium accumulation by 46%, 32% and 29% respectively. Larvae exposed to 10 μM Cd with 1 mM Ca co-treatment had a significant reduction in cadmium accumulation ($p < 0.001$). Co-treatment with 5 mM Ca resulted in a significant decrease in cadmium accumulation at each cadmium exposure concentration, relative to cadmium exposure alone ($p < 0.01$ for 1 μM Cd; $p < 0.001$ for 5 and 10 μM Cd).

3.2.3 Time course of concentration-dependent expression of *hsp70/eGFP* over the 96 h co-exposure period

To further investigate the potential for calcium co-treatment to ameliorate the effects of sublethal cadmium exposure, exposures were conducted using *hsp70/eGFP* transgenic larvae. These zebrafish larvae were observed at regular intervals throughout the 96 h exposure period for stress-responsive induction of the reporter gene. Specifically, expression of *hsp70/eGFP* in the olfactory epithelium was of greatest interest given the previously described effects of sublethal cadmium exposures on the olfactory system of larval zebrafish. As detailed in section 3.1.3, expression of the reporter gene in the developing lens is due to constitutive expression of *hsp70* and not due to cadmium or calcium exposure.

Zebrafish larvae exposed to either calcium concentration were identical to untreated control larvae at all observation time points (Figure 3.12), with only constitutive lens expression of the *hsp70/eGFP* reporter gene observed in these groups. In comparison, exposure to 1 μM Cd

Figure 3.12 Time course of *hsp70/eGFP* expression in transgenic zebrafish larvae during continuous exposure to calcium. Whole, living larvae were observed for expression of the reporter gene every 24 h of the 96 h exposure period. Exposure to 1 or 5 mM Ca did not induce expression of the reporter gene in any tissue throughout the 96 h period. Larvae in both treatment groups were similar to control at all observation points. Scale bar represents 250 μm.

Note: Lens expression of the reporter construct, observable in every larva, was due to constitutive *hsp70* expression as a part of normal lens development (Halloran et al., 2000; Blechinger et al., 2002b).

No Cd exposure

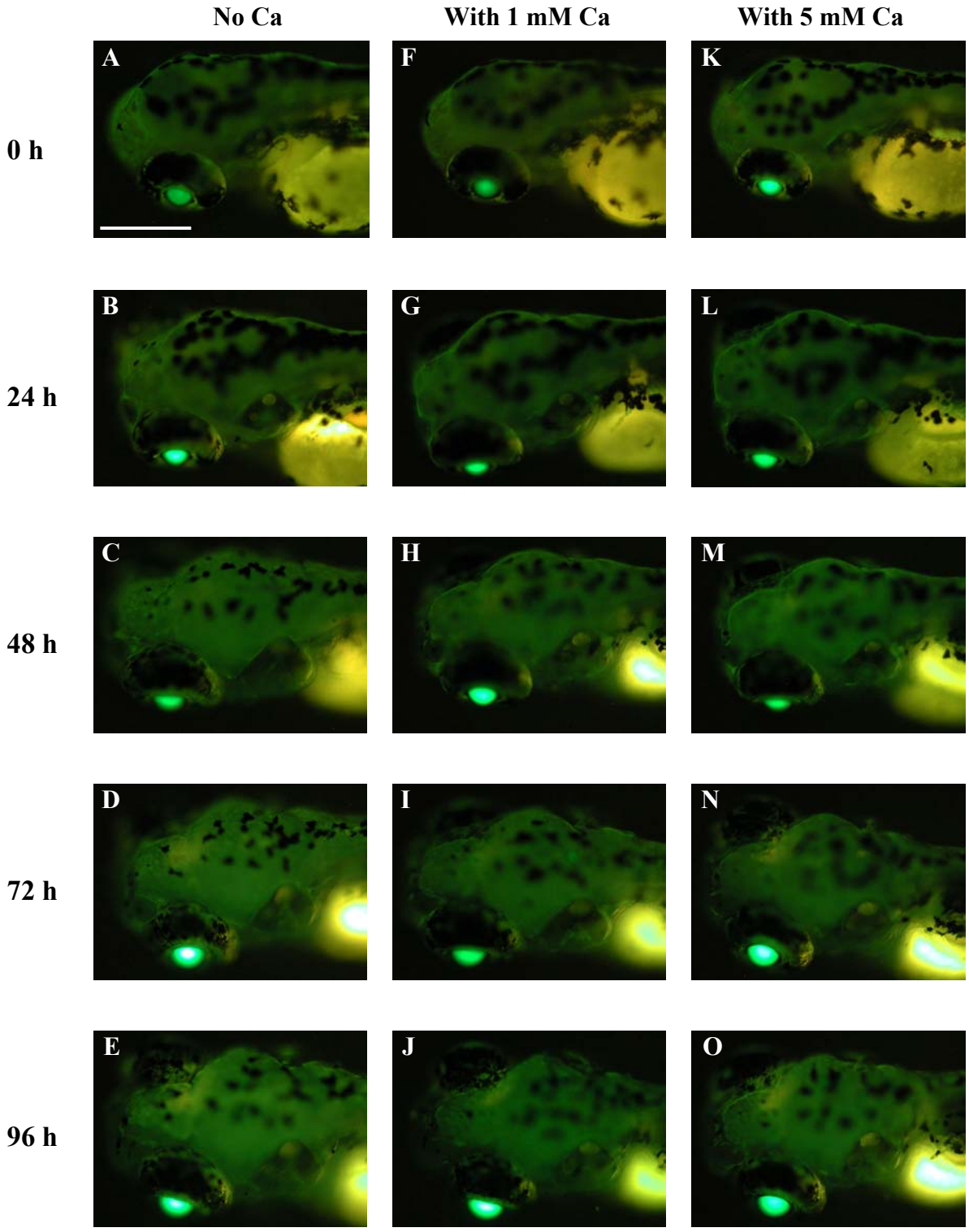
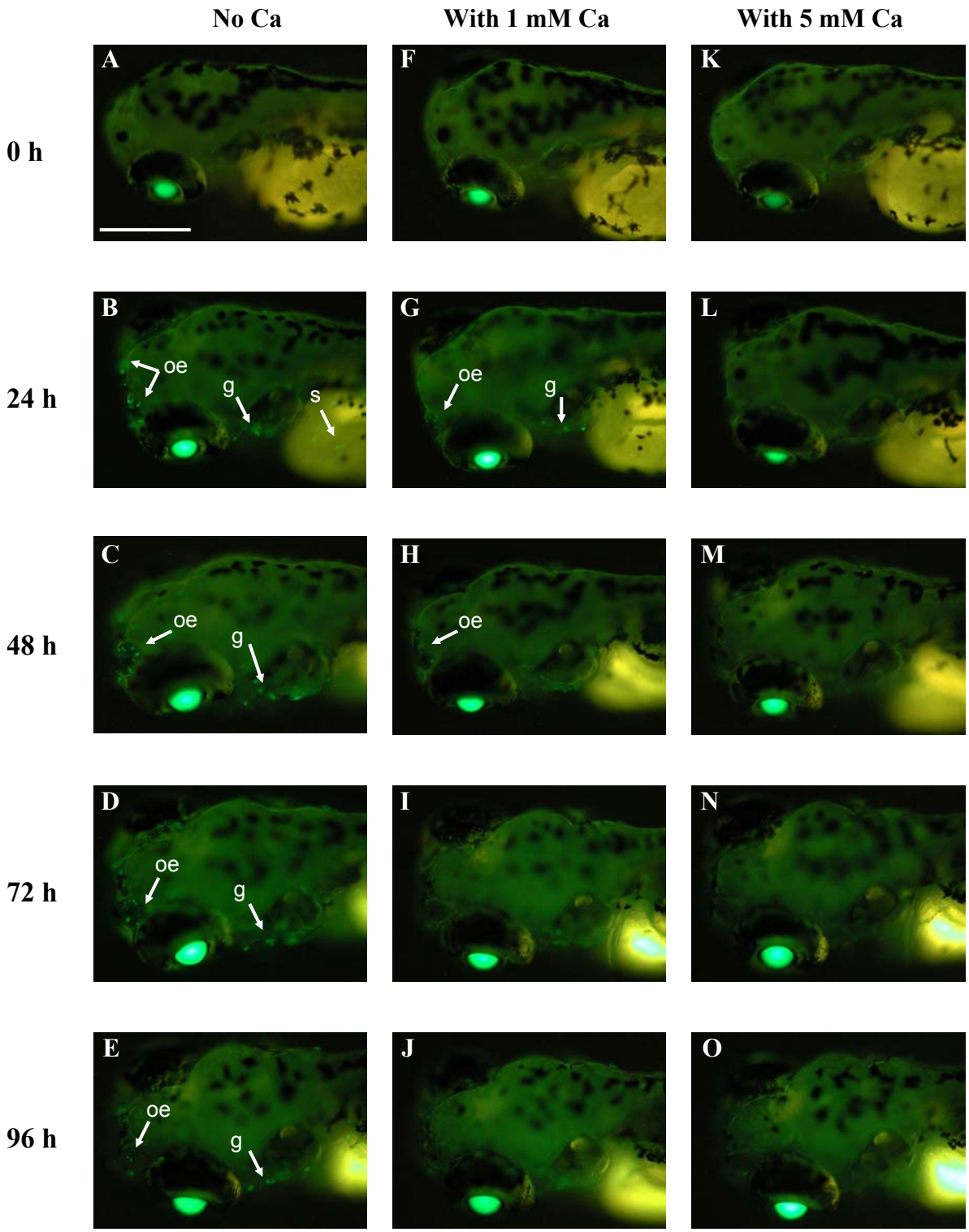


Figure 3.13 Time course of *hsp70/eGFP* expression in transgenic zebrafish larvae exposed to 1 μM Cd and with calcium co-treatments for 96 h. Whole, living larvae were observed for expression of the reporter gene every 24 h of the exposure period. Cadmium exposure induced relatively weak expression of *hsp70/eGFP* in the olfactory epithelium (panels B-E). Larvae co-treated with 1 mM Ca had reduced eGFP fluorescence in this tissue at 24 (panel G) and 48 (panel H) h of the exposure period, and no expression of the reporter gene was observed in the olfactory epithelium at 72 (panel I) and 96 (panel J) h of the exposure period. No cadmium-induced expression of *hsp70/eGFP* was observed in larvae co-treated with 5 mM Ca at any observation point (panels L-O). Scale bar represents 250 μm. g – gill, oe – olfactory epithelium, s – skin

Note: Lens expression of the reporter construct, observable in every larva, was due to constitutive *hsp70* expression as a part of normal lens development (Halloran et al., 2000; Blechinger et al., 2002b).

1 µM Cd exposure



resulted in reporter gene expression in the olfactory epithelium and gills (Figure 3.13, panels B-D). Co-exposure to 1 μM Cd with 1 mM Ca reduced eGFP fluorescence in the olfactory epithelium and gill tissue at 24 and 48 h of exposure (Figure 3.13, panels G and H), and no expression of the reporter gene was observed in these tissues at 72 or 96 h (Figure 3.13, panels I and J). When 1 μM Cd exposures were combined with the high calcium co-treatment, no stress-responsive *hsp70/eGFP* gene expression was observed at any time point (Figure 3.13, panels K-O).

Transgenic zebrafish larvae exposed to 5 μM Cd exhibited strong reporter gene expression in the olfactory epithelium at 24 and 48 h of exposure (Figure 3.14, panels B and C), with the expected decrease in eGFP fluorescence at 72 and 96 h of exposure (Figure 3.14, panels D and E). Co-exposure of 5μM Cd with the low calcium treatment resulted in a small decrease in reporter gene expression in the olfactory epithelium (Figure 3.14, panels G-J). This reduction in eGFP fluorescence compared to exposure to cadmium alone was most evident at the 48 and 72 h observation points (Figure 3.14, compare panels C and H, D and I). Co-treatment with 5 mM Ca largely diminished stress-responsive *hsp70/eGFP* gene expression in the olfactory epithelium (Figure 3.14, panels L-O), compared to 5 μM Cd exposure alone. With the high calcium co-treatment, very few cells of the olfactory epithelium were expressing the reporter gene at each time point of the 96 h exposure period.

Larvae exposed to 10 μM Cd also had the expected expression pattern of *hps70/eGFP* in the olfactory epithelium, with strong eGFP fluorescence at 24 and 48 h (Figure 3.15, panels B and C) and a noticeable decrease at 72 and 96 h (Figure 3.15, panels D and E). Addition of 1 mM Ca as a co-treatment, resulted in a minor decrease in eGFP fluorescence in the olfactory epithelium at each of the observation points (Figure 3.15, panels G-J). With the high calcium co-

Figure 3.14 Time course of *hsp70/eGFP* expression in transgenic zebrafish larvae exposed to 5 μM Cd and with calcium co-treatments for 96 h. Whole, living larvae were observed for expression of the reporter gene every 24 h of the exposure period. Cadmium exposure induced relatively strong expression of *hsp70/eGFP* in the olfactory epithelium at 24 (panel B) and 48 (panel C) h, with reduced eGFP fluorescence at 72 (panel D) and 96 (panel E) h of the exposure period. Larvae co-treated with 1 mM Ca had reduced eGFP fluorescence in the olfactory epithelium at each observation point of the exposure period (panels G-J). A further decrease in reporter gene expression was observed in larvae co-treated with 5 mM Ca (panel L-O). Scale bar represents 250 μm. g – gill, oe – olfactory epithelium, s – skin

Note: Lens expression of the reporter construct, observable in every larva, was due to constitutive *hsp70* expression as a part of normal lens development (Halloran et al., 2000; Blechinger et al., 2002b).

5 µM Cd exposure

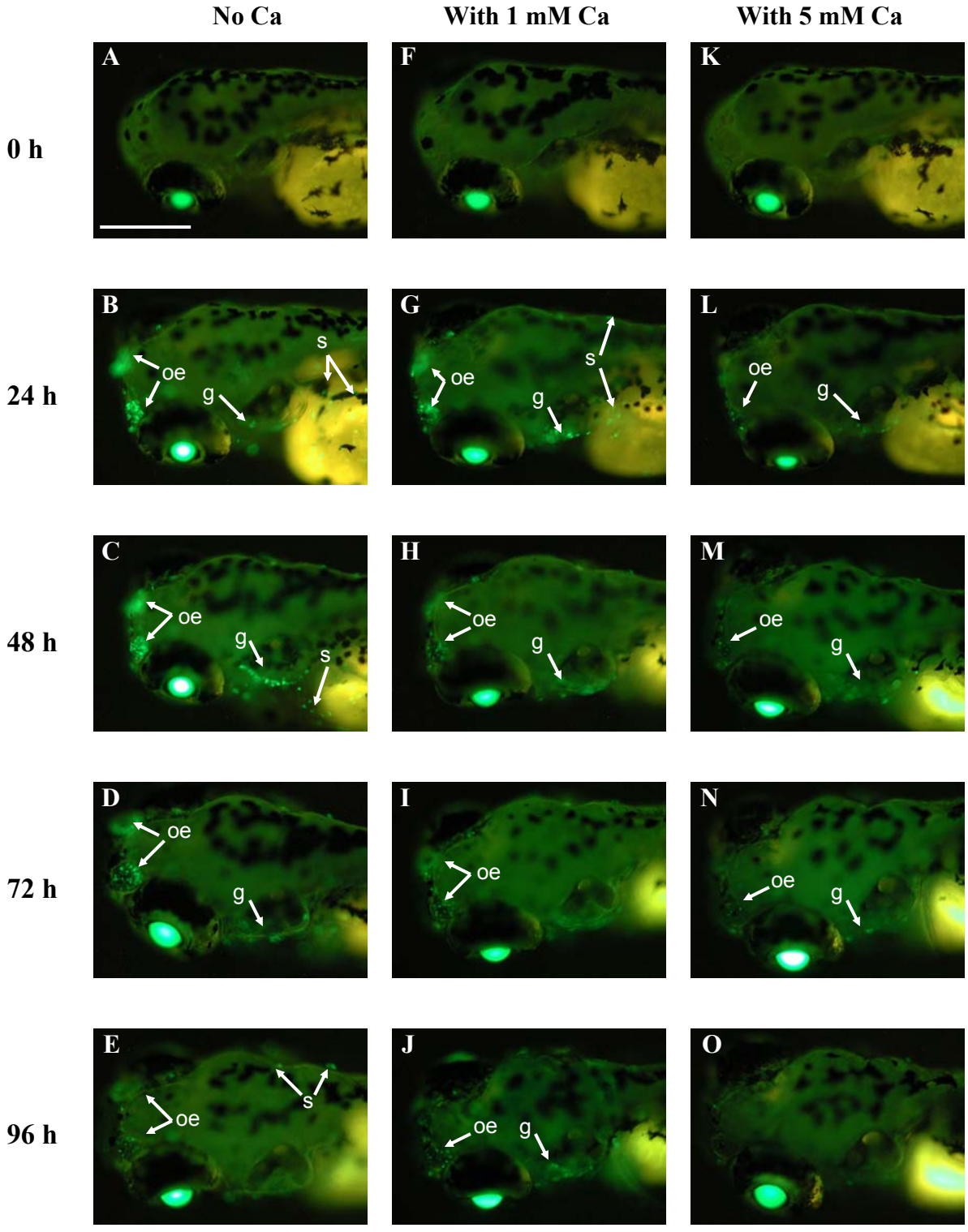
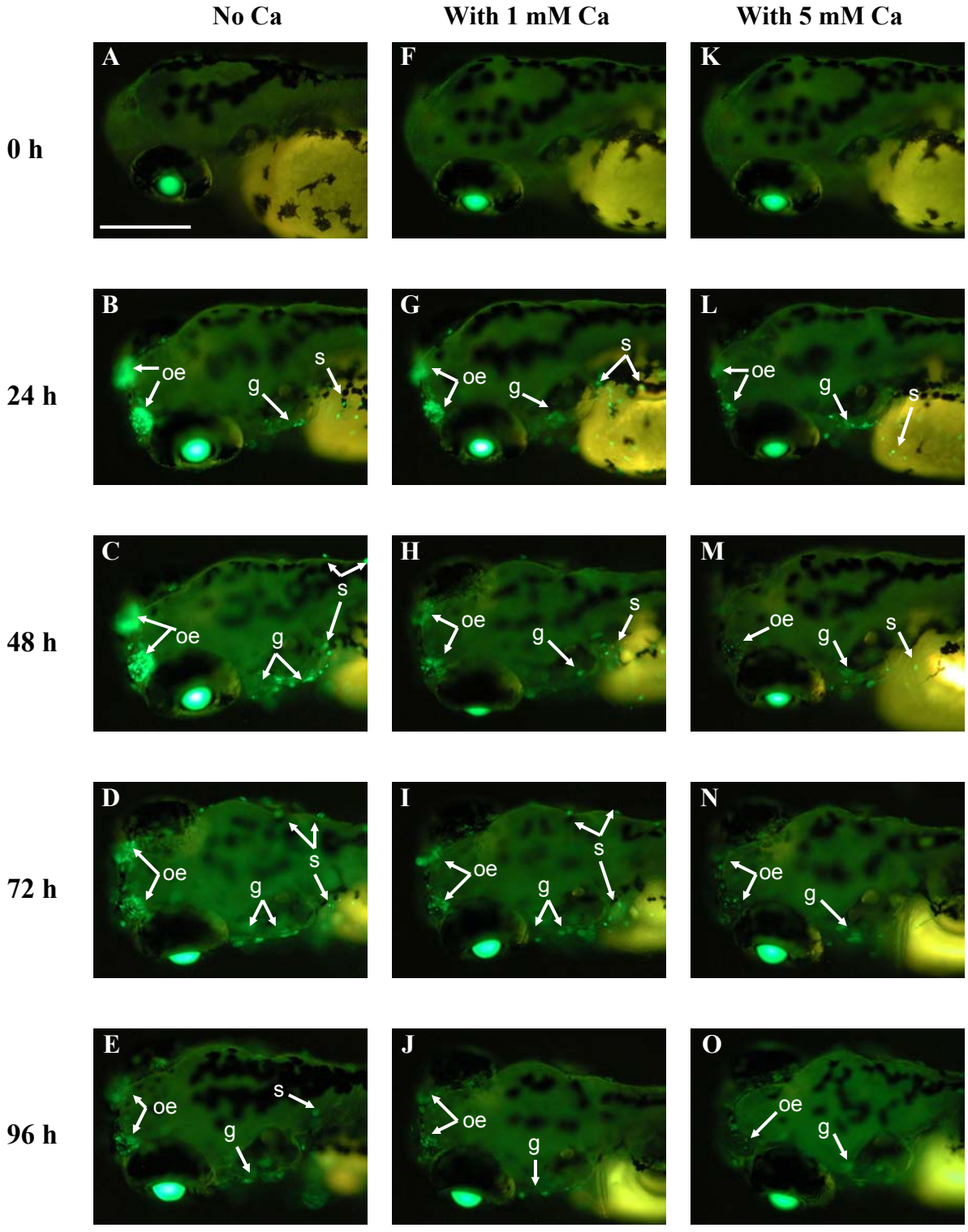


Figure 3.15 Time course of *hsp70/eGFP* expression in transgenic zebrafish larvae exposed to 10 μM Cd and with calcium co-treatments for 96 h. Whole, living larvae were observed for expression of the reporter gene every 24 h of the exposure period. Cadmium exposure induced relatively strong expression of *hsp70/eGFP* in the olfactory epithelium at 24 (panel B) and 48 (panel C) h, with noticeably reduced eGFP fluorescence at 72 (panel D) and 96 (panel E) h of the exposure period. Larvae co-treated with 1 mM Ca had reduced hsp70/eGFP fluorescence in the olfactory epithelium at each observation point of the exposure period (panels G-J). A further decrease in reporter gene expression was observed in larvae co-treated with 5 mM Ca (panel L-O). Scale bar represents 250 μm. g – gill, oe – olfactory epithelium, s – skin

Note: Lens expression of the reporter construct, observable in every larva, was due to constitutive *hsp70* expression as a part of normal lens development (Halloran et al., 2000; Blechinger et al., 2002b).

10 µM Cd exposure

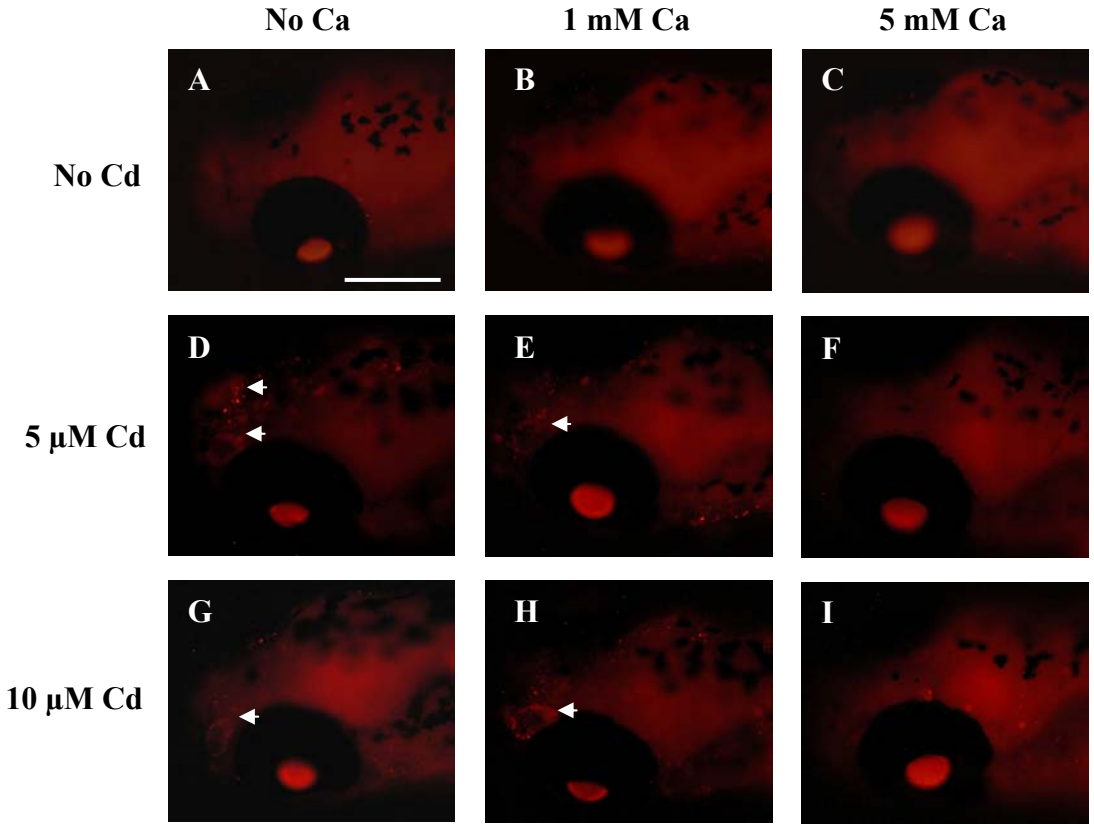


treatment, expression of the reporter gene was greatly reduced in the olfactory epithelium (Figure 3.15, panels L-O), as only a small number of cells were fluorescing at each observation point. The effect of increasing calcium co-treatment on reducing expression of *hsp70/eGFP* in the olfactory epithelium was most evident at the 24 and 48 h time points (Figure 3.15, panels B, G, L and C, H, M, respectively). Cadmium exposure alone resulted in very strong induction of the reporter gene, while co-treatment with calcium abated *hsp70/eGFP* expression in the olfactory epithelium.

3.2.4 Cell death in the olfactory epithelium following cadmium and calcium co-exposure

To further investigate the impact of additional calcium on the effects of cadmium exposure, cell death (TUNEL) assays were performed following the 96 h exposure period. As described previously (section 3.1.4), 1 μM Cd exposures did not result in any TUNEL labeling in the olfactory epithelium of larval zebrafish; thus, this cadmium exposure group was not included in the cell death analysis. Many TUNEL-positive cells were observed in the olfactory epithelium of larvae exposed to 5 and 10 μM Cd (Figure 3.16, panels D and G). Similar to previous results, the anatomical location and circular organization of TUNEL-positive cells directly corresponds to expression of the *hsp70/eGFP* reporter gene observed in the olfactory epithelium of the transgenic larvae. Extensive cell death, as indicated by TUNEL labeling, was also observed in the olfactory epithelium of larvae exposed to 5 and 10 μM Cd with 1 mM Ca co-treatment (Figure 3.16, panels E and H). Alternatively, of larvae in the high calcium co-exposure groups, no olfactory cell death was observed at 5 or 10 μM Cd with 5 mM Ca co-treatment group (Figure 3.16, panels F and I). No TUNEL-positive cells were evident in larval zebrafish from unexposed and calcium only treatment groups (Figure 3.16, panels A-C).

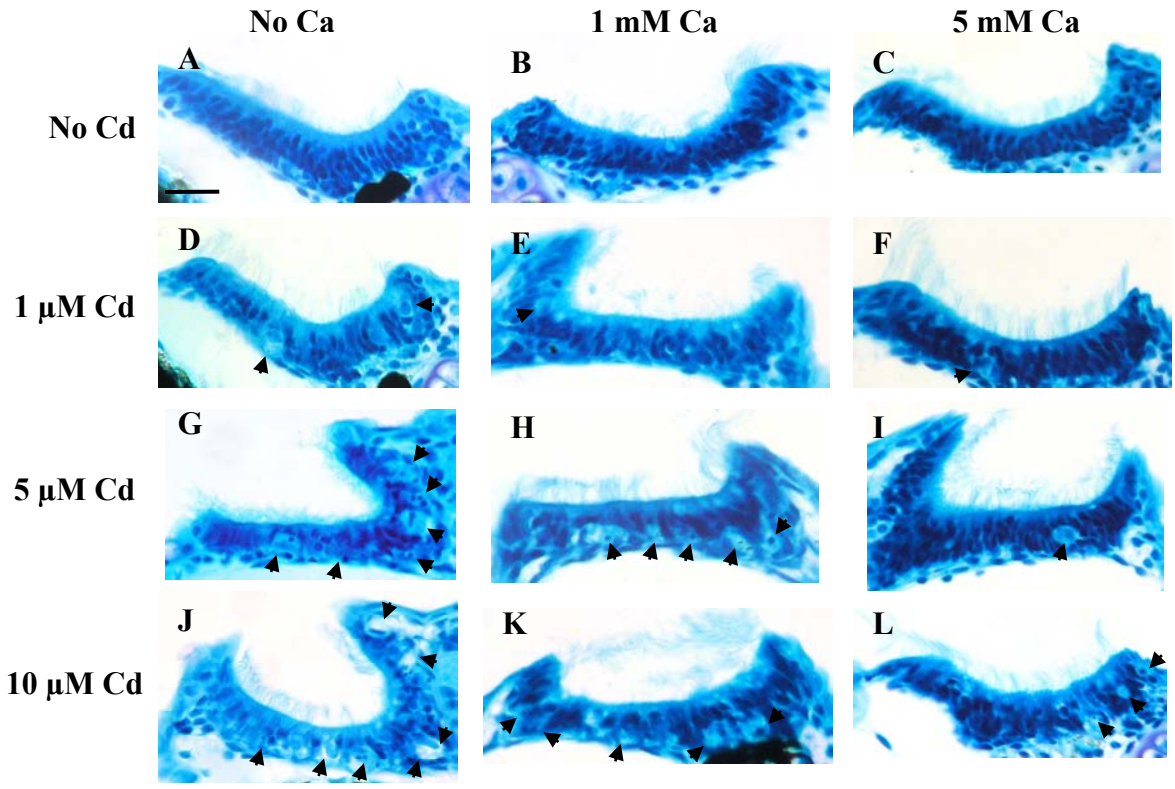
Figure 3.16 Cadmium-induced cell death in the olfactory epithelium of larval zebrafish following 96 h exposure to cadmium, calcium, or in combination as assessed using the TUNEL assay. Many TUNEL-positive nuclei were observed in the olfactory epithelium of larvae exposed to 5 (panel D) or 10 (panel G) μM Cd, as indicated by arrows. Co-treatment of 1 mM Ca with 5 (panel E) and 10 (panel H) μM Cd, also resulted in olfactory epithelium cell death, while no cell death was observed in this tissue with 5 mM Ca co-treatment (panels F and I). No cell death was observed in larvae exposed to calcium only (panels B and C). Scale bar represents 250 μm.


No Ca
1 mM Ca
5 mM Ca
No Cd
5 µM Cd
10 µM Cd
A
B
C
D
E
F
G
H
I

3.2.5 Histological effects of cadmium and calcium co-exposure on the olfactory epithelium

Histopathological analysis was performed on all treatment and control groups to further assess the modulating effects of calcium co-treatment on cadmium-induced effects in the olfactory epithelium of zebrafish larvae (Figure 3.17). Untreated larvae had densely packed layers of cells forming the olfactory epithelium (Figure 3.17, panel A). Similar to these control larvae, no overt histological effects were observed in the sensory epithelium of larvae exposed to calcium only (Figure 3.17, panels B and C). Histological alterations observed in larvae exposed to cadmium only were similar to those described in section 3.1.5. Briefly, the extent of histopathological alterations in the olfactory epithelium was dose-dependent, with irregular areas of cell loss and round, darkly stained structures were often associated with these areas. With increasing calcium co-exposure, the histological effects were diminished. The olfactory epithelium of larvae exposed to 1 μM Cd and 1 or 5 mM Ca had little histological alteration (Figure 3.17, panels E and F), although they were not wholly identical to control larvae. With co-exposure to 1 mM Ca, fewer of these histological abnormalities were observed in the 5 and 10 μM Cd exposure groups (Figure 3.17, panels H and K). With the high calcium co-treatment, only a few histopathological effects were evident in the olfactory epithelium at the higher cadmium exposures of 5 and 10 μM Cd (Figure 3.17, panels I and L).

Figure 3.17 Histopathology of zebrafish larvae olfactory epithelium following 96 h exposure to cadmium, calcium, or in combination. Abnormal histopathology in the olfactory epithelium was observed in all larvae exposed to cadmium as areas of cell loss (panels D-L; arrowheads). Often associated with these areas are small, round, darkly stained structures. The degree of histopathological effects increased with cadmium exposure (compare panels down a column). Co-treatment with 5 mM Ca reduced the extent of abnormal histopathology in the olfactory epithelium (panels F, I, L). Exposure to calcium alone did not alter the olfactory epithelium (panels B and C). Scale bar represents 20 μm.


No Ca
1 mM Ca
5 mM Ca
No Cd
1 µM Cd
5 µM Cd
10 µM Cd
A
B
C
D
E
F
G
H
I
J
K
L

3.2.6 Behavioural analysis of zebrafish larvae following cadmium and calcium co-exposure

To further examine the ameliorating effect of calcium treatment on the impact of cadmium exposure, behavioural L-cysteine aversion tests were conducted at the end of the 96 h exposure period to assess the function of the olfactory system. The results of the behaviour trials are presented in Figure 3.18, with each panel representing a given cadmium exposure group. Typical aversion responses to the L-cysteine stimulus for unexposed and calcium only treated fish were similar to those described for control larvae in section 3.1.7. Also, aversion behaviours of larvae exposed to cadmium, whether alone or in combination with calcium, were also similar to those previously described for cadmium exposed fish in section 3.1.7. The average proportion of larvae responding to the L-cysteine odorant was greater than 0.8 for the untreated and calcium only control exposure groups (i.e. no cadmium; Figure 3.18, panel A). The differences between these groups were not found to be statistically significant, indicating that 1 and 5 mM calcium exposures, in the absence of cadmium, did not have an effect on function of the olfactory system.

In each of the cadmium only treatment groups the average proportion of larvae responding to the L-cysteine stimulus was significantly less than the response in untreated control larvae (i.e. no cadmium or calcium; $p < 0.05$ for 1 μM Cd, $p < 0.001$ for 5 and 10 μM Cd). For each of the cadmium exposure groups a trend of increasing aversion response with increasing calcium co-treatment was observed; however, the interaction between cadmium and calcium was not quite significant ($p<0.0689$). This increase in aversion response was significant in the 5 μM Cd exposure groups ($p < 0.05$; Figure 3.18, panel C). Although the observed increase in the average proportion of larvae responding to the L-cysteine stimulus was not significant with increasing calcium co-treatment in the 1 and 10 μM Cd exposure groups (Figure

3.18, panels B and D, respectively), the general trend was evident. No statistically significant differences were found between responses to the distilled water control stimulus across the different exposure groups.

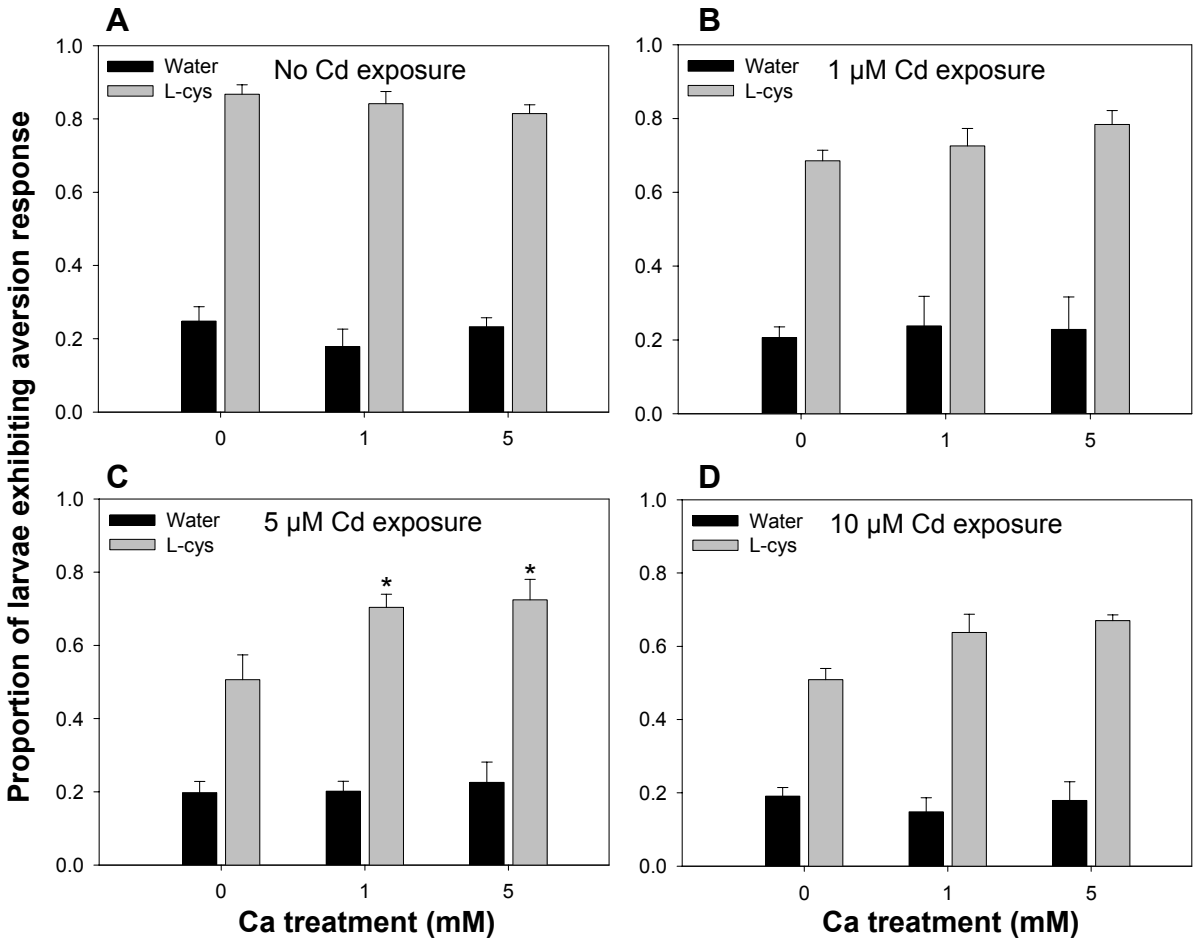
Figure 3.18 Effect of calcium co-treatment on behavioural responses of zebrafish larvae to L-cysteine following 96 h exposures to cadmium. Sublethal cadmium exposure decreased the aversion response to an L-cysteine stimulus, relative to a distilled water stimulus, indicating olfactory impairment in larval zebrafish (see Figure 2.1 for schematic diagram of the assay). Data represents the average proportion of larvae (~15 larvae/replicate) exhibiting an aversion response to water stimulus (at least 4 replicates) or L-cysteine stimulus (at least 6 replicates). Error bars represent standard error. Two-way ANOVA with post-tests were used to analyze the effects of cadmium and calcium on aversion response to L-cysteine in zebrafish larvae. Summary of two-way ANOVA analysis is given below (Table 3.4).
* $p < 0.05$

Panel A – no cadmium exposure: calcium treatment on larvae not exposed to cadmium (i.e. unexposed control larvae) resulted in a slightly decreased response to L-cysteine that was not statistically significant. The average proportion of larvae responding to L-cysteine was greater than 0.8 in each group.

Panels B, C and D – cadmium exposure: Increasing calcium co-treatment resulted in an increased aversion response to L-cysteine by zebrafish larvae within a given cadmium exposure concentration. This observed increase was statistically significant in the 5 μM Cd exposure group (C). As expected, the average proportion of larvae responding to L-cysteine decreased with cadmium exposure concentration (compare between panels).

Table 3.4 Two-way ANOVA p-values for aversion to L-cysteine in zebrafish larvae exposed to cadmium, calcium, or in combination

Source of variation	p-value
Cadmium	<0.0001
Calcium	0.0062
Interaction	0.0689


A
B
C
D
Water
L-cys
No Cd exposure
1 μM Cd exposure
5 μM Cd exposure
10 μM Cd exposure
Proportion of larvae exhibiting aversion response
Ca treatment (mM)
0.0
0.2
0.4
0.6
0.8
1.0
0
1
5
*

Chapter 4

4. Discussion

The central objective of this research project was to investigate the effects of cadmium exposure on zebrafish larvae and, more specifically, the effect of these cadmium exposures on the olfactory system at the cellular and functional levels. Additionally, the potential for activation of the heat shock stress response pathway in the olfactory system to act as an early indicator of sensory effects was explored using a transgenic line of *hsp70/eGFP* zebrafish. Finally, the potential for calcium to ameliorate the adverse effects of cadmium on zebrafish larvae at the whole organism, cellular, and functional levels was investigated.

4.1 Cadmium exposures with zebrafish larvae

This study had a mechanistic perspective which necessitated careful consideration in choosing cadmium concentrations for the larval zebrafish exposures. Namely, a range of exposure levels that would induce various degrees of adverse effects on the olfactory system, but have limited or no effect on other systems, was desired. The range of cadmium concentrations (0.5-10 μM Cd) utilized in this study was chosen as these concentrations lie below the previously reported 96 h LC_{50} for zebrafish larvae (Blechinger et al., 2002a). This range of exposure concentrations is higher than those used by some other authors; however, compared to other fish species, zebrafish are relatively insensitive to cadmium as higher concentrations are required to produce adverse effects. This observation is evident when comparing 96 h LC_{50} values for cadmium in larval stage fish of different species (Table 4.1). Only reported values for larval stage fish were used for comparison to limit any confounding factors due to age or developmental stage differences, as larvae are more sensitive than individuals in either

embryonic (Blechinger, 2002; Hallare et al., 2005b) and juvenile (Buhl, 1997) stages of development. Zebrafish larvae are at least one order of magnitude less sensitive to cadmium compared to almost all the species of fish surveyed, including tilapia and fathead minnows which are often used as model fish for toxicological studies. Rainbow trout and chinook salmon larvae are close to two orders of magnitude more sensitive than zebrafish larvae. In comparison, larval common carp are more tolerant to cadmium exposure and have a reported LC_{50} that is roughly half that of zebrafish larvae.

Table 4.1 Comparison of 96 h LC_{50} values for cadmium toxicity in larval stage fish

Species	96 h LC_{50} (µM Cd)	Relative sensitivity (zebrafish LC_{50}/species LC_{50})	Reference
Common carp	38.3	0.5	Suresh et al., 1993
Zebrafish	18.8	1.0	Blechinger et al., 2002a
Tilapia	1.82	10.3	Hwang et al., 1995
Fathead minnow	0.71 – >1.3	<14.4 – 26.5	Hall et al., 1986
Bonytail	1.32	14.2	Buhl, 1997
Razorback sucker	1.24	15.2	Buhl, 1997
Colorado squawfish	0.69	27.1	Buhl, 1997
Rainbow trout	0.27	69.6	Van Leeuwen et al., 1985
Chinook salmon	0.23	81.7	Hamilton and Buhl, 1990
Australian crimson spotted rainbow fish	0.07	269	Williams and Holdway, 2000

Unfortunately, systematic comparisons of relative sensitivities to cadmium between zebrafish and other species of fish have not been reported. However, a decreased sensitivity for zebrafish compared to other fish species has been reported for several other compounds (Dave et al., 1981; Elonen et al., 1998; Van den Belt et al., 2003). Elonen et al. (1998) suggested that the apparent differences in sensitivity may be related to differences in early life stage development, accumulation in sensitive tissues, and physiology. Also, greater tolerance to dioxin was related to a shorter period of time for development from hatching to feeding and potentially greater rates of elimination (Elonen et al., 1998). Compared to trout and other salmonids, the rapid development of zebrafish may partially explain the decreased sensitivity to cadmium.

Additionally, as zebrafish are a tropical fish they are maintained at a higher temperature (28.5°C) compared to fish found in Canadian waterways (e.g. rainbow trout ~12°C). This may result in differences in physiology and metabolism leading to potentially greater rates of elimination and reduced sensitivity. This factor may also partially explain the relatively high LC_{50} reported for common carp larvae, as the fish were maintained at 29°C in the study (Suresh et al., 1993). In comparison, other studies with have maintained carp at a much cooler 7°C (de Conto Cinier et al., 1997, 1999) or a more moderate 20°C (Reynders et al., 2006; Huang et al., 2007); however LC_{50}'s for carp were not reported in these studies.

4.1.1 Gross toxic effects of cadmium exposure on zebrafish larvae

Zebrafish larvae exposed to cadmium displayed the expected dose-response effects. The incidence of mortality was less than 50%, as the tested concentrations were below the reported LC_{50} of 18.8 μM Cd (Blechinger et al., 2002a). Generally, the incidence of mortality increased with cadmium concentration; however responses were similar for 5 and 10 μM Cd (Figure 3.2, panel A). Also, a significant incidence of mortality was only observed in these exposure groups, relative to the control group. Similarly, in the initial investigations of cadmium toxicity to zebrafish larvae, an increased percent mortality relative to control larvae was not observed in larvae exposed to 1 μM Cd and below, but was observed in larvae exposed to 2.5 μM Cd and above (Blechinger, 2002). The specific cause of death in fish due to short-term or acute cadmium exposure has not been directly investigated, though may be similar to lethal effects reported for mammals. In mammals, acute cadmium inhalation or ingestion causes severe endothelial damage leading to fluid loss from the capillaries, edema, ischemia, and tissue necrosis (WHO, 1992b; ATSDR, 2000). Cadmium-induced death in fish may also be the result

of several adverse effects including damage to gill tissue (Karlsson-Norrgren et al., 1985; Wong and Wong, 2000), interference with calcium uptake (Verbost et al., 1988, 1989), and altered ion homeostasis (Pratap et al, 1989).

Incidences of edema and trunk abnormalities also increased with cadmium exposure concentration. For these non-lethal morphological effects, all cadmium concentrations tested had a significant increase in effects relative to unexposed larvae (Figure 3.2, panel B). Interestingly, morphological effects were observed in about 50% of the larvae exposed to 5 μM Cd, which was higher than the previously reported EC_{50} of 1.7 μM Cd for zebrafish larvae (Blechinger et al., 2002a). This difference was unexpected given the observed agreement with the previously reported LC_{50} from the same study. However, the upper 95% confidence interval for Blechinger et al's (2002a) reported EC_{50} was greater than 4 μM Cd, suggesting that the incidences of morphological effects observed in this study were not inconsistent with the previous findings. A significant incidence of morphological effects was observed in the lowest cadmium exposure group of 0.5 μM. This observation is similar to the initial investigations of cadmium toxicity in larval zebrafish in which larvae exposed to 0.2 μM Cd were identical to control larvae, while edema and trunk abnormalities were observed in 15% of larvae exposed to 0.5 μM Cd (Blechinger, 2002). Based on the present study and Blechinger (2002), the 0.5 μM Cd exposure level represents a LOEC (lowest observed effects concentration) for zebrafish larvae over a 96 h exposure period.

A common non-lethal effect of cadmium exposure was development of trunk abnormalities in the larval zebrafish (described in section 3.1.1 and Figure 3.1, panels C and D). Cadmium-induced trunk deformities in embryonic and larval stage fish have been described for several species including zebrafish (Cheng et al., 2000; Blechinger et al., 2002a; Chan and

Cheng, 2003; Chow and Cheng, 2003; Hallare et al., 2005a), Atlantic salmon (Rombough and Garside, 1982), common carp (Witeska et al., 1995), and Australian crimson spotted rainbow fish (Williams and Holdway, 2000). Spinal deformities in embryonic zebrafish due to cadmium exposure during the gastrulation and segmentation stages of development (5 to 28 hpf) have been attributed to altered muscle development (Cheng et al., 2000; Chow and Cheng, 2003), cell death in the neural tube (Chan and Cheng, 2003), and failure of the notochord to extend to the tail region (Chow and Cheng, 2003). The morphologies of the deformities presented in the above studies were dissimilar to the lordosis and kyphosis observed in larval zebrafish in this study, likely due to differences in developmental stage at the time of exposure. Spinal malformations and lesions in the spinal columns of fish due to cadmium exposure have also been related to decreased calcium levels in the vertebral column (WHO, 1992b), and mice pups exposed to cadmium in utero had skeletal malformations and reduced bone ossification (Padmanabhan and Hameed, 1990). However, the cadmium-induced trunk abnormalities observed in larval stage zebrafish in this study are likely not due to effects of cadmium on ossification of the vertebrae. Trunk abnormalities were observed in zebrafish larvae at ~5-7 days post fertilization, yet ossification in the vertebral column does not begin until ~7-9 days post fertilization (Fleming et al., 2004). The mechanism by which cadmium exposure induces trunk deformities in larval stage zebrafish is at present unknown, and is likely due to adverse effects in the developing vertebrae or notochord.

4.1.2 Whole-body cadmium accumulation in zebrafish larvae with regard to exposure length and concentration

Routes of waterborne cadmium uptake by larval zebrafish are likely similar to that of fully developed fish, including facilitated diffusion through Ca^{2+}-channels in the gill epithelium (Verbost et al., 1987, 1989; Wicklund Glynn et al., 1994) and uptake by the olfactory system (Tjalve et al., 1986). Cadmium accumulation by zebrafish larvae in the present study increased with exposure concentration and exposure length (Figure 3.3). Larvae exposed to 10 μM Cd had the greatest amount of whole-body cadmium accumulation, and accumulation increased over the entire time course in all exposure groups. A similar pattern of increasing cadmium accumulation with time and exposure concentration was reported for Atlantic salmon alevins (larval stage; Rombough and Garside, 1982). Interestingly, at the three lower exposure levels of 0.5, 1, and 5 μM Cd, the overall time course of cadmium accumulation was quite similar despite a difference of up to an order of magnitude in exposure concentrations. However, the incidences of mortality and morphological effects were greater in the 5 μM Cd exposure groups even though cadmium accumulation was similar (Figure 3.2). Likewise, zebrafish embryos exposed to 2 and 10 mg Cd/L had differences in mortality rates, but similar amounts of cadmium accumulation over a 48 h exposure period (Hallare et al., 2005a). The divergence between cadmium accumulation and gross effects is further highlighted when comparing the 5 and 10 μM Cd exposure groups. The incidences of mortality and non-lethal effects were not significantly different between these groups; however, larvae from the higher exposure group had an almost 30% higher average cadmium accumulation compared to the 5 μM Cd group. Together these observations suggest that cadmium body burdens are not accurate predictors of gross toxic effects and death in zebrafish larvae.

Due to the small size of zebrafish larvae, whole-body cadmium accumulation was determined as opposed to tissue-specific measurements which can be more informative regarding uptake, distribution, and elimination of a compound. For example, greater cadmium accumulation was found in gill tissue compared to muscle tissue of common carp larvae for short-term acute exposures (4.3 mg/L for 4 days), however the reverse was observed with longer subacute exposure (0.86 mg/L for 30 days; Suresh et al., 1993); cadmium accumulation in other tissues, such as liver or kidney, was not reported. In the same study, fingerling carp accumulated the most cadmium in the gills, slightly less in the liver and kidney and least in the muscle with short-term acute 17.1 mg/L for 4 days) and longer subacute (3.42 mg/L for 30 days) exposures (Suresh et al., 1993). In comparison, a study of the time course of cadmium accumulation in rainbow trout found that levels of cadmium in gill tissue were constant and similar in fish exposed to 25 and 50 μg/mL over a 3 week period, while kidney levels were at least 5 fold greater and increased with exposure length and concentration (Melgar et al., 1997). Interestingly, cadmium did not accumulate in the liver until the second week and levels were less than measured in the kidney (Melgar et al., 1997). A similar time course of cadmium accumulation was reported by Hollis et al. (2001) for rainbow trout exposed to 3 μg/mL for 30 days, suggesting that in rainbow trout equilibrium is quickly established in the gills, thus maintaining constant cadmium levels in the site of entry. Furthermore, cadmium appears to preferentially accumulate in the kidney before sequestration or storage in the liver. A completely dissimilar time course of cadmium accumulation was observed in tilapia exposed to 10 μg/mL for 35 days (Pratap and Wendelaar Bonga, 2004). Tilapia initially accumulated cadmium mainly in the kidney and gills and levels in these tissues decreased substantially from day 14 to 35 of the exposure period (Pratap and Wendelaar Bonga, 2004). Additionally, the level of cadmium in the

liver was highest early in the exposure period (4 days) and while levels did decrease in this tissue, it was not to the same extent as in the kidney and gills (Pratap and Wendelaar Bonga, 2004). These observations suggest that tilapia are able to eliminate cadmium at a greater rate than cadmium uptake during a continuous exposure. These examples illustrate that patterns of cadmium accumulation cannot be generalized and species differences in uptake, distribution, and elimination are widely variable.

The studies of tissue-specific cadmium accumulation described above mainly focused on accumulation in the gills, liver and kidneys, which is typical for most work in this research area. However, fish also accumulate cadmium from surrounding waters via the olfactory system (Tjalve et al., 1986; Scott et al., 2003). Furthermore, on a size basis, the olfactory system accumulates similar or even greater amounts of cadmium as the liver and kidney (Scott et al., 2003), suggesting that cadmium readily enters this sensory system. The impact of cadmium on the olfactory system will be addressed further in the following sections.

4.1.3 Induction of the heat shock stress response in zebrafish larvae following cadmium exposure

Previous investigations with *hsp70/eGFP* transgenic zebrafish larvae exposed to cadmium (Blechinger et al., 2002a) and arsenic (Salisbury, 2006) have established this line of transgenic larvae as reliable indicators of cell-specific and dose-dependent toxic effects. Short-term 3 h exposure to acute concentrations up to 125 µM Cd identified the olfactory epithelium along with gill tissue, skin, liver, pronephros (kidney), and lateral line as target tissues of acute cadmium exposure in zebrafish larvae (Blechinger et al., 2002a). In the present study, the sensitivity of the olfactory system to cadmium was further investigated using lower

concentrations and a longer exposure duration. Induction of the transgene was observed in the olfactory epithelium in a dose-dependent fashion at all cadmium concentrations tested (Figure 3.4). As repeated observations of the transgenic larvae are easily performed, a time course of *hsp70/eGFP* expression was generated to ascertain the effect of cadmium on the olfactory system with respect to exposure length. A pattern of initial activation of the *hsp70/eGFP* transgene expression in the first 48 h followed by an unexpected decrease of eGFP fluorescence for the last 48 h was observed in the olfactory epithelium for all cadmium concentrations tested. This decrease in eGFP fluorescence was unexpected as the larvae were continuously exposed to cadmium for the entire 96 h exposure period. Additionally, the time course of cadmium accumulation (Figure 3.3) showed an increased body burden with exposure length, indicating that uptake was greater than elimination over the entire exposure period. Although measurements of cadmium accumulation were whole-body and not olfactory tissue-specific, it is likely that an increasing total body burden correlates to an increasing accumulation in all tissues. I hypothesized that this observation was likely due to either recovery of the cells from cadmium-induced stress and/or death of the affected cells. In an individual cell, these outcomes are mutually exclusive, but in a whole tissue both cell recovery and cell death can occur. Additionally, induction of the hsp70 stress response did vary widely between treatment groups. At lower cadmium concentrations the stress on individual cells may have been non-lethal allowing recovery to predominate in the tissue. However, at higher cadmium concentrations, the stress response may have been insufficient for survival in a majority of the affected cells leading to cell death in the olfactory epithelium.

Supporting the idea of cell recovery is the very nature of the hsp70 stress response. Hsp70 is a molecular chaperone involved in protein repair that is expressed in cells as a defense

mechanism following stress. When chaperoning requirements in a cell are satisfied expression of *hsp70* is down-regulated (reviewed in Morimoto et al., 1992; Iwama et al., 1998; Katschinki, 2004). Additionally, the heat shock stress response has been demonstrated by several authors to be transient for a variety of stressors (e.g. Krone and Heikkila, 1988; Matranga et al., 2002; Wada et al., 2005). During a continuous exposure to heat shock levels of *hsp70* transcripts were found to rapidly increase then decrease in zebrafish embryos (Lele et al., 1997) and *Xenopus laevis* tadpoles (Krone and Heikkila, 1988). In the latter study, Northern blot analysis revealed that after a continuous 21 h heat shock *hsp70* mRNA levels had returned to background amounts (Krone and Heikkila, 1988). Similarly, acid stress in sea urchin coelomocytes greatly increased levels of hsp70 after a 2 h stress, while much lower protein levels were observed at 4 and 6 h of continuous acid stress (Matranga et al., 2002). This transient hsp70 stress response has also been reported for cadmium-induced stress (Wada et al., 2005; Ali et al., 2003). For example, HepG2 cells transfected with a plasmid containing a luciferase reporter gene under the control of a *hsp70* promoter were continuously exposed to a range of non-lethal cadmium concentrations and had a strong dose-dependent increase in luciferase activity (a measure of *hsp70* gene expression) in the first 12-15 h of exposure followed by a continuous decrease throughout the remainder of the 48 h exposure period (Wada et al., 2005). These examples illustrate that cells can adapt to various stressors and that *hsp70* gene expression may be down-regulated in cells that have adapted to stress, even if the stress is still present in the system. Also, these observations may explain the observed decrease in eGFP fluorescence in the olfactory epithelium of transgenic zebrafish larvae, especially at lower cadmium concentrations where the observed stress response was relatively weak.

Evidence to support the alternative hypothesis that the observed decrease in eGFP fluorescence in the olfactory epithelium was due to cell death is more indirect. Firstly, cadmium exposure has been linked to olfactory impairment in fish (Baker and Montgomery, 2001; Scott et al., 2003) and humans (Sulkowski et al., 2001; Mascagni, et al., 2003; reviewed in Gobba, 2003, 2006). However, the mechanism(s) by which cadmium exposure leads to the observed sensory impairment has not been well characterized and death of olfactory cells may be involved. Cadmium exposure has been demonstrated to induce cell death in many cell types (Piechotta et al., 1999; Lopez et al., 2003; Mao et al., 2007), including the olfactory epithelium (Blechinger et al., 2007). Further experiments were conducted to investigate this issue of cell recovery and/or cell death in the olfactory epithelium and are discussed in the following sections.

4.1.4 Degeneration of the olfactory epithelium in zebrafish larvae following cadmium exposure

Damage to the olfactory epithelium of larval zebrafish following cadmium exposure was correlated with substantial decreases in eGFP fluorescence using TUNEL assays for cell death and histological analysis. Transgenic larvae exposed to 5 and 10 µM Cd had the greatest expression of *hsp70/eGFP* at 48 h of exposure, followed by an obvious decrease in eGFP fluorescence at 72 and 96 h of exposure. TUNEL assays indicated that cells of the olfactory epithelium were dying in larvae exposed to 10 µM Cd for 72 and 96 h and in larvae exposed to 5 µM Cd for 96 h (Figure 3.5). Further histological analysis revealed considerable damage to olfactory tissue at 72 and 96 h of exposure in larvae from both cadmium treatment groups (Figure 3.7). Of the two techniques, histological analysis was decidedly more sensitive. For example, minor alterations to the olfactory epithelium were observed using histology in larvae

exposed to 5 μM Cd for 72 h and 1 μM Cd for 96 h and in comparison, whereas TUNEL staining was not observed in larvae from these groups. At the higher cadmium exposure concentrations of 5 and 10 μM, cell death and histological analyses suggested that the observed decrease in eGFP fluorescence was due at least in part to cell death. Cadmium-induced cell death in the olfactory epithelium has been reported in studies using substantially higher exposure concentrations (Stromberg et al., 1983; Blechinger et al., 2007). Severe necrosis was observed in juvenile fathead minnows following 96 h acute exposures to over 100 μM Cd (Stromberg et al., 1983), and many TUNEL positive cells were observed in zebrafish larvae exposed to 125 μM Cd for 3 h (Blechinger et al., 2007).

In comparison, larvae exposed to 0.5 and 1 μM Cd had relatively weak induction of the *hsp70/eGFP* transgene in a small number of cells compared to the observations at the higher cadmium exposures. Although a decrease in eGFP fluorescence was observed in larvae from these exposure groups, minor or no adverse effects were observed in the olfactory epithelium via histological analysis. The observed decrease in eGFP fluorescence in larvae exposed to 0.5 μM Cd was likely due to cell recovery as neither TUNEL nor histological analysis revealed any cellular-level effects. In the 1 μM Cd exposure group, no TUNEL staining was observed, but minor alterations were detected in the olfactory epithelium after 96 h exposure using histology. This exposure level represents a transition between 0.5 and 5 μM Cd, and a combination of cellular loss and recovery in the olfactory epithelium is likely responsible for the observed decrease in eGFP fluorescence.

Compared to cadmium, the effects of copper exposure on fish olfactory systems at the cellular level is well described in the literature. Chronic copper exposure in rainbow trout was found to cause adverse effects throughout the olfactory system, not just at the surface of the

olfactory epithelium, as degeneration of receptor cells, axons forming the olfactory nerve, and axon terminals in the olfactory bulb was observed (Juillard et al., 1993). Histopathological alterations were dose-dependent in the olfactory epithelium of yearling rainbow trout exposed to 20 or 40 µg/L Cu for 40 weeks (Saucier and Astic, 1995). At the higher copper concentration, the olfactory epithelium was disorganized and had many vacuoles throughout the epithelium; these vacuoles were attributed to hypertrophy of goblet cells and likely contained mucus. At the lower copper concentration, slight disorganization was observed due to hypertrophy and hyperplasia of goblet cells. The authors suggest that changes in goblet cell number and size were to increase mucus production as a primary defense to prevent copper from entering the tissue (Saucier and Astic, 1995). Additionally, degeneration of the olfactory epithelium was observed after much shorter 4 day exposures in tilapia exposed to 20, 40, and 100 µg/L Cu (Bettini et al., 2006). At the higher 100 µg/L Cu exposure level, cells of the olfactory epithelium appeared looser, had a loss of cell connections, and had areas of cell loss, while at the lower copper concentrations the effects were less severe. These histological alterations described by Bettini et al. (2006) are similar to the effects of cadmium exposure observed in the present study with zebrafish larvae.

To further characterize the effects of cadmium exposure on the olfactory system of zebrafish larvae, topographical analysis was performed using scanning electron microscopy. No changes to the surface of the olfactory epithelium were observed in larvae exposed to 0.5 or 1 µM Cd for 72 or 96 h. Larvae from the lower cadmium exposure groups were comparable to unexposed control larvae, corresponding to the observations of cell death and tissue histology. Also similar to TUNEL and histological analyses, adverse effects were observed in the higher concentration cadmium exposure groups that had strong induction of the *hsp70/eGFP* transgene

followed by a marked decrease in eGFP fluorescence. Loss of sensory cilia on the surface of the olfactory epithelium and changes to the shape of the olfactory pit were observed in larvae exposed to 5 and 10 μM Cd for 72 (Figure 3.7) and 96 (Figure 3.8) h. Interestingly, the long, non-sensory kinocilia appeared to be unaffected by cadmium exposure. A similar effect was reported for chinook salmon and rainbow trout exposed to 25 to 300 μg/L Cu for 4 h, as fewer ciliated and microvillar dendrites were observed with copper exposure while the number of non-sensory ciliated dendrites was unchanged at all copper concentrations (Hansen et al., 1999). Additionally, non-sensory ciliated cells were unaffected by chronic copper exposure while receptor neurons died throughout the olfactory epithelium (Julliard et al., 1993, 1996). In comparison, adverse effects on both sensory and non-sensory cilia were reported for fish exposed to 15 μg/L inorganic mercury for 24 h (de Oliveira Riberio et al., 2002), and to 3mg/L sodium dodecylbenzene sulphonate (a surfactant) for 5 days (Zeni and Stagni, 2002). The observed disparity in effects on the ciliated cell types may be related to species differences and/or mode of action of the toxicant on the cells. Regardless, the sensory cells appear to be highly sensitive to chemical exposure, as effects were observed in these cells with each toxicant, and after short-term pulse exposures and long chronic exposures.

4.1.4.1 Impairment of olfactory sensory function in zebrafish larvae following cadmium exposure

With the evidence that sublethal cadmium exposures cause adverse effects on the olfactory epithelium of zebrafish larvae, behavioural assays were performed to determine whether these cadmium exposures were impairing an olfactory-dependent response. This assay is based on olfactory-mediated perception of L-cysteine leading to avoidance behaviours in

zebrafish larvae and was originally used to identify an olfactory mutant from a genetic-screen (Vitebsky et al., 2005). In the present study, response to L-cysteine by zebrafish larvae was significantly reduced following sublethal cadmium exposure for 96 h (Figure 3.9). Furthermore, the decrease in aversion to L-cysteine increased with cadmium concentration corresponding to the observed dose-dependent effects on the olfactory epithelium. More specifically, behavioural responses to the odorant were greatly reduced at the higher cadmium exposure levels of 5 and 10 μM. This was not unexpected as larvae in these exposure groups had severe histological alterations in and loss of many sensory cilia from the olfactory epithelium, both of which would contribute to a loss of sensory function. Larvae exposed 1 μM Cd also exhibited a reduced olfactory response, but not to the same extent as the higher exposure groups, which was expected based on the minor histological alterations observed in the olfactory epithelium at this cadmium concentration. At the lowest exposure concentration of 0.5 μM Cd the observed response to L-cysteine was decreased compared to control larvae but not found to be significant, also corresponding to the results obtained from the histological and topographical analyses for this exposure group.

Olfactory impairment in fish due to metal exposure during early life stages has been reported for cadmium (Blechinger et al., 2007; Kusch et al., 2007) and copper (Saucier et al, 1991; Carreau and Pyle, 2005). Both studies involving cadmium were conducted with zebrafish, but had completely different exposure regimens. Blechinger et al. (2007) reported deficits in olfactory-mediated anti-predator avoidance behaviours in juvenile zebrafish (4-6 weeks old) that had been briefly exposed to 5, 25, and 125 μM Cd during the larval stage. These acute short-term exposures induced the *hsp70* stress response and caused cell death in the olfactory epithelium of the zebrafish larvae, which were associated with the observed olfactory

impairment in juveniles (Blechinger et al., 2007). In comparison, Kusch et al. (2007) found that the anti-predator response due to olfactory-mediated perception of alarm substance was almost eliminated in juvenile zebrafish that had been continuously exposed to 20 µg/L Cd from shortly after fertilization to two weeks before behaviour testing (fish were in clean water for the period between exposure and testing). The observed loss of olfactory function was suggested to be due to cadmium-induced necrosis or altered development of the olfactory system (Kusch et al., 2007); however these possibilities were not specifically investigated in the study. Additionally, Carreau and Pyle (2006) reported that exposure of fathead minnows to 10 µg/L Cu during the first five to seven days of embryonic development prior to hatching, followed by a return to clean water, was sufficient to cause deficits in olfactory-mediated behaviours once these fish reached the juvenile stage at 84 to 96 days. In comparison, rainbow trout exposed to 22 µg/L Cu from hatch for 8 months had impaired behavioural responses to olfactory stimuli (Saucier et al., 1991). The specific effects of copper on the developing olfactory system in these studies are not known as analysis of olfactory system at the cellular or tissue level was not conducted by either Carreau and Pyle (2006) or Saucier et al. (1991), though the latter suggested olfactory dysfunction was due degeneration of the olfactory epithelium. Together with the present study on the effects of cadmium on the olfactory system of larval zebrafish, these studies indicate that the olfactory systems of developing fish are vulnerable to metal pollution and that deficits in olfactory function can persist long after the exposure has ended.

Cadmium exposure in juvenile fish has also been demonstrated to impair olfactory function (Baker and Montgomery, 2001; Scott et al., 2003). Banded kokopu exposed to 0.5 and 1 µg/L Cd for 48 h did not exhibit a preference for adult pheromones which are known olfactory-perceived attractants (Baker and Montgomery, 2001). Also, exposure to 2 µg/L Cd for 7 days

eliminated anti-predator avoidance behaviours in rainbow trout, whereas dietary exposures at a higher cadmium concentration did not affect these behaviours indicating that olfactory dysfunction was the result of waterborne cadmium exposures (Scott et al., 2003). Furthermore, the observed sensory impairment following waterborne exposures was related to cadmium uptake in the olfactory system, including the olfactory rosette, nerve, and bulb (Scott et al., 2003). Previous studies have demonstrated that cadmium in the surrounding waters enters olfactory sensory neurons, induces metallothionein expression, and is transported along the axons as a cadmium-metallothionein complex to the axon terminals in the olfactory bulb where it accumulates (Tjalve et al., 1986; Gottofrey and Tjalve, 1991; Tallkvist, et al., 2002). Metallothionein expression has been observed in the olfactory epithelium of zebrafish embryos and larvae following exposure to 5 μM Cd (Chen et al., 2007), suggesting that cadmium also enters this tissue in early life stage zebrafish.

In the present study, the observed effects on the olfactory system of larval zebrafish were likely the result of cadmium entering the olfactory sensory neurons and inducing expression of *hsp70* as a stress response. The observed decrease in olfactory-mediated behaviour in zebrafish larvae observed as a result of cadmium exposures was likely due at least in part to the adverse effects of cadmium on the olfactory epithelium at the cellular and tissue level. Similarly, loss of olfactory sensitivity in rainbow trout and chinook salmon due to copper exposure has been linked to morphological alterations in the olfactory epithelium, including loss of sensory cilia (Hansen et al., 1999) and histological alterations (Saucier and Astic, 1995). These studies suggest that loss of sensory function reported for wild fish in metal-contaminated lakes (McPherson et al., 2004) may be due to direct effects of metal pollutants on the olfactory epithelium. Since the olfactory system has a central role in many essential fish behaviours, including feeding,

reproduction, and predator avoidance, impairment of sensory function would have adverse effects on fish populations.

4.1.5 Comparison of endpoints used to assess cadmium toxicity in zebrafish larvae

In this study, several endpoints were measured to assess cadmium toxicity in zebrafish larvae including: mortality, non-lethal morphological effects, heat shock stress response, cell death, histopathological alterations, topographical changes, and behavioural effects. For each endpoint the LOEC is summarized below in Table 4.2.

Table 4.2 Summary of LOEC values by endpoint used to assess cadmium toxicity in zebrafish larvae

Endpoint	96 h LOEC (µM Cd)
Mortality	5
Non-lethal morphological effects	0.5
hsp70/eGFP expression in olfactory epithelium	0.5
TUNEL-labeling for cell death	5
Histological alterations	1
Topographical changes (loss of sensory cilia)	5
Behaviour effects (reduced aversion to L-cysteine)	1

As demonstrated in Table 4.2, the relativity sensitivity between the endpoints was variable. For effects in the olfactory system, the most sensitive endpoint was expression of *hsp70/eGFP* in the olfactory epithelium. Expression of the reporter gene in this tissue was induced at a concentration below which effects were observed using other techniques, indicating the sensitive nature of this transgenic reporter system. Of note, although expression of *hsp70/eGFP* in zebrafish larvae was the most sensitive endpoint in this study, this system likely would not be useful for routine biomonitoring. The Canadian Water Quality Guideline for protection of aquatic life is 0.017 µg Cd/L (~0.15 nM Cd; CCME, 2002), which is several orders of magnitude below which cadmium-induced transgene expression was observed in zebrafish larvae. Even

though the zebrafish model system appears to be relatively insensitive to cadmium (see section 4.1), this system may be most suitable for mechanistic investigations like the present study.

4.2 Modulation of the effects of cadmium on larval zebrafish by calcium

Interactions between cadmium and calcium are well known, and the toxic effects of cadmium exposure are strongly associated with disturbances in calcium metabolism and homeostasis (reviewed in WHO, 1992a). Additionally, several authors have demonstrated that calcium reduces cadmium toxicity in fish species including zebrafish (Meinelt et al., 2001), trout (Pascoe et al., 1986; Hansen et al., 2002), striped bass (Wright et al., 1985), and mummichog (Gill and Epple, 1992). In the present study, the potential for calcium to ameliorate the effects of sublethal cadmium exposures on zebrafish larvae was examined by exposing larvae to cadmium and an excess of calcium in combination.

4.2.1 Gross toxic effects of cadmium exposure on zebrafish larvae modulated by calcium

The toxic effects of cadmium exposure in fish are associated with hypocalcemia which stems from competitive inhibition of Ca^{2+}-ATPases by cadmium on the basolateral membrane of the gill epithelia, thereby limiting calcium uptake to the bloodstream (Verbost et al., 1988, 1989). Since the inhibition is competitive, the extent of cadmium-induced hypocalcemia is ameliorated with increased water calcium (Pratap et al., 1989). In the present study, excess calcium exerted a protective effect on the occurrence of gross toxic effects of cadmium exposure in zebrafish larvae. The incidences of mortality and non-lethal morphological effects of cadmium exposure on zebrafish larvae were reduced with increasing calcium co-treatment (Figure 3.10). Exposures

conducted with the high calcium co-treatment had a dramatic effect as incidences of mortality and morphological effects were reduced to levels almost as low as those observed in the control groups of larvae.

Similarly, excess calcium has been demonstrated to greatly reduce the acute toxicity of cadmium in several fish species. Near 100% survival was observed in early life stage zebrafish exposed to cadmium concentrations as high as 9.3 mg/L Cd (~83 μM Cd) with 2 mM Ca for 144 hours (from fertilization to larval stage; Meinelt et al., 2001). Also, the incidence of mortality in striped bass larvae exposed to 5 or 10 μg/L Cd with 125 mg/L Ca (~3 mM Ca) for 5 days was not different than control groups, while a substantial reduction in mortality with 8 mg/L Ca (~0.2 mM Ca) was only observed at the 5 μg/L Cd exposure level (Wright et al., 1985). Neither of these studies reported observations of cadmium-induced morphological effects, such as edema or trunk abnormalities, though these effects would be expected in larval stage fish exposed to cadmium. Additionally, the observed mortality was almost an order of magnitude lower in juvenile rainbow trout exposed to 2 μg/L Cd with 770 or 1200 μM Ca for 30 days compared to mortality rates observed in cadmium exposures without additional calcium (Hollis et al., 2000). Further, this reduced mortality was correlated with reduced cadmium accumulation in gills, liver, and kidney (Hollis et al., 2000). For the present study, the observed decreases in gross toxic effects of cadmium in zebrafish larvae exposed in combination with excess calcium were likely the result of excess calcium alleviating hypocalcemia and potentially a reduction in cadmium accumulation.

4.2.2 Effect of calcium on whole-body cadmium accumulation in zebrafish larvae

Previous studies have demonstrated that cadmium movement across the gill epithelia, the main site of cadmium uptake in fish, occurs via voltage-independent calcium channels on the apical surface of chloride cells and diffusion through the basolateral membrane to systemic circulation (Verbost et al., 1987, 1989; Wicklund Glynn et al., 1994). Competition between cadmium and calcium for uptake occurs at the calcium channels on the apical surface of the gill epithelia, as increase in waterborne calcium and calcium channel blockers have been demonstrated to decrease cadmium uptake (Verbost et al., 1987; Wicklund Glynn et al., 1994; Niyogi and Wood, 2004). As expected, in the present study cadmium accumulation in zebrafish larvae over the 96 h exposure period was decreased with calcium co-treatment (Figure 3.11). Additionally, a greater reduction in cadmium accumulation was observed with the high calcium co-treatment compared to the low calcium co-treatment. The largest percent decrease of close to 50% was observed in larvae exposed to 1 μM Cd with 5 mM Ca, representing a 5000-fold excess of calcium compared to cadmium treatment alone. A similar reduction was observed in tilapia larvae exposed to cadmium for 48 h also with a 5000-fold calcium excess (only the one cadmium and calcium combination was used in the study; Wu et al., 2007). Furthermore, this inverse relationship between cadmium accumulation and ambient waterborne calcium concentration has also been described for black sea bream (Zhang and Wang, 2007) and minnows (Wicklund and Runn, 1988).

Interestingly, additional calcium did not limit cadmium accumulation to the extent that was anticipated based on the large decreases in mortality and morphological effects observed in the calcium co-treatment groups (Figure 3.10). These observations suggest that excess calcium likely had a predominant effect maintaining calcium homeostasis while having a lesser effect on

limiting cadmium uptake at the gills. For example, Wu et al. (2007) found the reduction in whole-body calcium content in tilapia larvae exposed to cadmium and calcium was less than in larvae exposed to cadmium only. Further, the authors suggested that the improved calcium homeostasis accounted for the lack of growth inhibition in larvae exposed to cadmium and calcium compared to the decreased growth observed in larvae exposed to cadmium alone (Wu et al., 2007). Additionally, the dissimilarity between the effects of cadmium exposure in combination with calcium on gross indicators of toxicity and cadmium body burdens further suggests the poor predictive quality of the whole-body burdens, as described previously in section 4.2.1. Pascoe et al. (1986) also reported that the reduced toxicity of cadmium to rainbow trout in hard water compared to soft water (i.e. different calcium levels) was not related to differences in cadmium accumulation.

Measurements of whole-body cadmium accumulation in zebrafish larvae after a 96 h exposure quantified by GFAAS (Figure 3.3) and ICP-MS (Figure 3.11) were different, with roughly twice as much cadmium measured at each exposure concentration using ICP-MS. This discrepancy may be due to the differences in sample preparation and analytical techniques, but also a result of differences in the larvae used in the two investigations. The experiments were conducted over two years apart, thus the breeding stock adult zebrafish were not the same, and the genetic background of the larvae may have been different. Differences in cadmium uptake between batches of fish have been reported by other authors (Wicklund Glynn et al., 1994). For example, in a series of studies from Hollis et al. (1999, 2000, 2001) involving cadmium accumulation in juvenile rainbow trout using the same exposure conditions and analytical techniques, the amount of cadmium accumulated in the gill and liver varied by as much as 4 fold, yet the pattern of tissue-specific cadmium accumulation was similar between the studies.

4.2.3 Modulation of cadmium-induced heat shock response in zebrafish larvae by calcium

In the initial investigations of the effect of cadmium on the olfactory system of larval zebrafish, the effects observed at the cellular and functional levels were attributed to cadmium gaining entry to the olfactory epithelium, as cadmium has been demonstrated to accumulate in the olfactory system (Tjalve et al., 1986; Scott et al., 2003). The exact route of cadmium entry to the olfactory epithelium from surrounding waters has not been established. However, I hypothesized that in a scenario similar to the gills, cadmium enters the olfactory system by exploiting calcium uptake systems. A large number of calcium channels are associated with the cilia of sensory neurons that extend into the environment, each of which may serve as a potential site for cadmium entry to the olfactory system. Cadmium may enter these cells via the cyclic-nucleotide gated channels associated with olfactory sensory perception. Additionally, cadmium could antagonize any other calcium uptake system present on the surface of the olfactory epithelium to enter the tissue.

Regardless of the exact nature of the route, if cadmium does enter the cells via calcium systems it was expected that co-treatment with calcium would limit cadmium uptake thereby having protective effects on the olfactory system. Transgenic *hsp70/eGFP* zebrafish larvae exposed to cadmium and calcium in combination exhibited a decreased activation of the reporter gene in the olfactory epithelium (Figure 3.13 - 3.15). Furthermore, a greater reduction in eGFP fluorescence was observed with the high calcium compared to the low calcium co-treatment. This dose-dependent inhibition suggests that cadmium is likely gaining entry to the olfactory epithelium via calcium systems, as increasing calcium in the exposure reduced the stress response; however, olfactory-specific cadmium uptake by zebrafish larvae was not measured in the present study. In a similar study using a strain of transgenic *C. elegans* with a β-

galactosidase reporter gene under the control of a stress inducible *hsp70* promoter, Guven et al. (1995) found that induction of the reporter gene by cadmium was reduced with increasing calcium concentrations. Additionally, the observed reduction in β-galactosidase activity was correlated with decreased cadmium accumulation by the nematodes in the presence of additional calcium (Guven et al., 1995). Interestingly, co-treatment with calcium did not completely abate induction of the stress response in the olfactory epithelium of zebrafish larvae exposed to cadmium. This suggests that cadmium was gaining entry to these cells despite the excesses of calcium, indicating that cadmium is readily acquired by the olfactory system. Scott et al. (2003) estimated that the olfactory system accumulates as much or more cadmium relative to the liver and kidney, well known target organs associated with cadmium exposure, on a size basis. Together, these observations suggest that the olfactory systems of fish are highly sensitive to cadmium exposure, even with the presence of factors that modulate the toxic effects of cadmium.

4.2.4 Modulation of cadmium-induced degeneration of the olfactory epithelium and sensory function in zebrafish larvae by calcium

The effects of cadmium and other metals on the olfactory systems of fish have been reviewed previously in section 4.1.4 and will not be discussed here to avoid repetition. The focus of this section will be to integrate the observations of the modulating effects of calcium co-treatment on cadmium-induced degeneration of the olfactory system and sensory function.

As expected from the reduced activation of the heat shock stress response, cell death and histological analyses revealed that increasing calcium co-treatment reduced the effects of cadmium on the olfactory system in zebrafish larvae (Figures 3.16 - 3.17). Interestingly, while calcium reduced the extent of histological alterations, the olfactory epithelium was not wholly

identical to control larvae, even at the lowest cadmium exposure level of 1 μM. This suggests that although calcium co-treatment may be limiting cadmium entry, cadmium exposure still induces changes in the olfactory epithelium, further highlighting the sensitive nature of this tissue. Additionally, induction of the *hps70/eGFP* reporter in the olfactory epithelium was again an early indicator of adverse effects at the tissue level. Further, greater numbers of dying cells and histological alterations corresponded to stronger expression of the reporter gene in the olfactory epithelium. As expected from the observations at the cellular level, calcium co-treatment had an ameliorating effect on olfactory sensory function. At all cadmium exposure concentrations tested, a greater aversion response to L-cysteine was observed with increasing calcium co-treatment (Figure 3.18). Although the observed increase in olfactory-dependent behaviours with calcium co-treatment was only found to be significant at the 5 μM Cd exposure level, this trend was evident in the 1 and 10 μM Cd exposure groups.

It was surprising that excess calcium did not result in a greater recovery of sensory function, especially in larvae with the high calcium co-treatment. While larvae exposed to cadmium in combination with 5 mM Ca exhibited a substantial amelioration of effects at the cellular level in the olfactory epithelium, this was not fully reflected in assessment of olfactory-dependent behaviour. The average proportion of larvae responding to L-cysteine in groups exposed to cadmium and 5 mM Ca was about 85% of the average proportion responding in the high calcium control group. This suggests that additional mechanisms of cadmium-induced impairment of olfactory sensory function were involved, as sensory impairment was observed with limited effects in the olfactory epithelium. Although eGFP fluorescence in the olfactory epithelium was noticeably reduced compared to larvae exposed to cadmium only, larvae exposed to 5 or 10 μM Cd with 5 mM Ca did still exhibit induction of the stress response in the olfactory

epithelium suggesting that cadmium had gained entry to the cells. Once cadmium enters olfactory sensory neurons, it induces expression of metallothionein and is transported to the axon terminals in the olfactory bulb as a complex with metallothionein (Tallkvist, et al., 2002). Thus, interference with axonal transport or cellular activities in the axon terminals could have lead to the observed sensory deficits without considerable effects in the olfactory epithelium. Alternatively, the less than expected increases in olfactory-dependent behaviour may have been due to direct action of cadmium on the surface of the olfactory epithelium. Deficits in sensory function in zebrafish exposed to cadmium only were related to loss of sensory cilia on the external surface of the olfactory pit (discussed in section 4.1.4). This loss of cilia may arise from direction action of cadmium on the cilia, and not necessarily due to changes inside the sensory neurons first. Unfortunately, topographical analysis using SEM of larvae exposed to cadmium and calcium was not available to confirm or counter this speculation. Reduced sensory cilia on the surface of the olfactory epithelium of larvae exposed to 5 or 10 μM Cd with additional calcium could account for the reduced aversion response to L-cysteine observed in larvae with limited histological alterations in the olfactory epithelium. In a study of chronic copper exposure on the olfactory system of rainbow trout, Julliard et al. (1993) did not observe any dendritic processes extended to the exterior even though the olfactory epithelium appeared to have recovered and regenerated during the long exposure period, suggesting that cilia and microvilli may be directly eliminated by copper in the surround waters. Additionally, adult zebrafish exposed to low concentrations of copper have reduced expression of sensory transduction proteins, such as odorant receptors, G-proteins, and ion channels (F. Tilton, personal communication). Decreased expression of these proteins due to contaminant exposure would likely reduce olfactory function in the absence of gross effects in the olfactory epithelium, and a

similar mechanism may be involved in the present study. Another possibility for the less than expected recovery of olfactory function could be due to the excess calcium treatment itself. A slight decrease in aversion response to L-cysteine was observed in larvae exposed to 1 and 5 mM Ca only compared to unexposed larvae (i.e. no calcium or cadmium; Figure 3.18, panel A). However the difference between groups was less than 5% suggesting that the calcium treatments were unlikely to be a main contributing factor to the less than expected recovery of olfactory function.

4.3 Utility of *hsp70/eGFP* transgenic zebrafish larvae as early indicators of toxic effects

In the present study, induction of the *hsp70/eGFP* reporter gene was found to be an early and sensitive indicator of potential olfactory impairment induced by cadmium exposure. Observation of eGFP fluorescence in the olfactory epithelium of transgenic zebrafish larvae following cadmium exposure was highly consistent, indicative of effects at the tissue level, and sensory impairment. Additionally, the severity of cellular effects and functional deficits corresponded with the strength of *hsp70/eGFP* reporter gene expression. For example, at the higher cadmium exposure concentrations, a very strong induction of *hsp70/eGPF* was observed in zebrafish larvae which correlated well with degeneration of the olfactory epithelium and decreased olfactory response. In the different analyses including induction of the stress response, larvae exposed to 1 μM Cd exposure represented a transition group between the severe effects observed at 5 and 10 μM Cd exposure levels and the limited effects at 0.5 μM Cd. Interestingly, although larvae exposed to 0.5 μM Cd were similar to control larvae in histological, topographical, and behavioural analyses of the olfactory system, induction of the *hsp70/eGFP* reporter gene was observed in the olfactory epithelium at this exposure level. Thus

the hsp70 stress response is activated in the olfactory system slightly below the threshold concentration required to alter the olfactory epithelium and cause deficits in olfactory behavior, making it suitable as an early indicator of cadmium concentrations that are approaching olfactory toxicity levels. Additionally, transgenic larvae co-exposed to cadmium and calcium had weaker expression of *hsp70/eGFP* in the olfactory epithelium, which was correlated with decreased cellular effects and reduced sensory deficits, further suggesting a link between activation of the reporter gene and potential olfactory impairment. This transgenic reporter gene system may prove advantageous for rapid assessment of compounds or mixtures for olfactory toxicity. For example, previous studies have demonstrated that arsenic exposure also induces expression of *hsp70/eGFP* in the olfactory epithelium of larval zebrafish (Salisbury, 2006), however it has yet to be determined if arsenic exposure leads to olfactory sensory impairment.

The GFP reporter systems are particularly advantageous as they do not require the addition of exogenous substrates for detection, and observations are made in whole, living organisms. Stable transgenic lines, such as the *hsp70/eGFP* zebrafish used in the present study, are preferential as the foreign DNA has stably inserted into the genome and, thus, the transgene is present in every nucleus. This ensures that the transgene will be expressed in a consistent and reproducible fashion between individual embryos/larvae, and between experiments. Although transient transgenics are much easier to generate, the foreign DNA construct is present in only a portion of the cells, and in different cells of different embryos, resulting in mosaic patterns of expression. . As the utility of transgenic reporter systems expands, they should find increased use in routine biomonitoring (Mutwakil et al., 1997), toxicological assessments (Blechinger et al., 2002a; Bhargav et al., 2008), and mechanistic investigations (Seok et al., 2007). In

particular, they should prove especially valuable in monitoring cell fate and function in individual organisms in real time following contaminant exposure.

Chapter 5

5. Conclusions

Several conclusions can be drawn from the present study on the effects of sublethal cadmium exposure on larval zebrafish:

a) Zebrafish larvae accumulate waterborne cadmium in a manner dependent on both exposure concentration and length of exposure. Additionally, uptake of cadmium is likely via calcium uptake systems as accumulation of cadmium was decreased with increasing calcium co-treatment. Further, whole-body burdens of cadmium in zebrafish larvae have limited predictive potential of the incidences of gross toxic effects due to cadmium exposure.

b) Gross toxic effects of cadmium exposure in zebrafish larvae are alleviated by additional calcium, indicating that lethality and morphological effects may arise from cadmium-induced hypocalcemia.

c) Sublethal cadmium exposures induce degeneration of the olfactory epithelium of larval zebrafish in a time- and dose-dependent manner. Cadmium-induced cell death, histological alterations, and loss of sensory cilia in the olfactory epithelium likely contribute to sensory impairment in zebrafish larvae following sublethal cadmium exposure.

d) Cadmium gains entry to the olfactory epithelium by antagonizing calcium uptake systems. Additional calcium can ameliorate the effects of cadmium exposure on the olfactory epithelium, but is not completely protective for cadmium-induced sensory deficits. Thus, sensory impairment in zebrafish larvae due to cadmium exposure is mediated through adverse effects not only on the olfactory epithelium.

e) Induction of the *hsp70/eGFP* reporter gene in the olfactory epithelium following cadmium exposure can serve as an early indicator of sensory impairment.

Chapter 6

6. Future directions

For this study, whole-body cadmium accumulation was measured; however, tissue-specific accumulation in the olfactory system would have been preferential. Knowing the relative levels of cadmium in the olfactory epithelium between the exposure groups and with the addition of calcium as a co-treatment would have lead to a greater understanding of how cadmium exposure was leading to sensory dysfunction. This information may be gained by using synchrotron technologies to quantify cadmium in semi-thin sections of the olfactory system. Also, whole larvae can be imaged using the synchrotron to determine where cadmium is located in the olfactory system, i.e. olfactory epithelium, axons, or olfactory bulb. Accumulation of cadmium in the olfactory bulb may be a contributor to sensory impairment, and synchrotron imaging would help to investigate this possibility.

To further characterize the route of entry for cadmium into the olfactory epithelium, short-term exposures could be conducted in the presence of specific calcium channels blockers. Following these exposures, the olfactory epithelium can be analyzed for cadmium uptake using synchrotron radiation, and transgenic *hsp70/eGFP* larvae could be screened for induction of the reporter gene in the olfactory epithelium following these exposures. This would help to establish the link between cadmium uptake into the olfactory system and activation of the heat shock response in this tissue.

To better resolve the issue of cell death or cell recovery following cadmium exposure, a system for determining the fate of the olfactory sensory neurons would be advantageous. For example, a transgenic line of zebrafish that expressed a reporter gene (e.g. a fluorescent protein) constitutively in the olfactory sensory neurons would be ideal. For example, the promoter for

olfactory marker protein (Celik et al., 2002) could be utilized to create a transgenic line of zebrafish that had labeled and easily identifiable olfactory sensory neurons. Cadmium exposures would be conducted similar to the present study, and the fate of the labeled cells could be monitored in real time. With this method, death of the sensory neurons would be evident in decreased numbers of cells expressing the reporter gene, and if a cell recovery pathway dominates, the number of labeled cells should be unchanged relative to control larvae.

Sublethal cadmium exposure induced expression of the *hsp70/eGFP* reporter gene in the olfactory epithelium. Additionally, expression of this reporter system is proposed as an early indicator of potential sensory deficits. Testing of more compounds for activation of *hsp70/eGFP* in the olfactory epithelium and assessment of olfactory function will determine the usefulness of this transgenic reporter system as such an indicator.

In the present study, repeated observations of *hsp70/eGFP* transgenic larvae revealed the pattern of strong eGFP fluorescence in the olfactory epithelium followed by a noticeable decrease with length of cadmium exposure. Recently, a method for quantification of stress-inducible eGFP fluorescence in zebrafish larvae has been reported (Seok et al., 2007). In the present study, quantification of eGFP with length of exposure would allow for better comparisons between exposure groups and observations times. Furthermore, sensory impairment could be related to a threshold level of eGFP fluorescence, improving the utility of the transgenic reporter system as an early indicator of sensory deficits. Additionally, the reduced induction of *hsp70/eGFP* in the presence of additional calcium would also be quantifiable, instead of reliance on qualitative observations.

More direct measurements of olfactory function would be useful to fully characterize the effects of cadmium on this sensory system. Electrophysiological analysis of the olfactory

epithelium is used to determine the responsiveness of neurons to different stimuli and the thresholds required for a response (Hara, 1992b). This type of analysis would determine the effects of cadmium exposure directly on sensory transduction. For example, cadmium may raise the threshold for a stimulus to be perceived, and the threshold may increase in a dose-dependent fashion. Additionally, electrophysiological analysis can be performed on the olfactory bulb to measure relay of signals from the olfactory nerve to the olfactory bulb (Hara, 1992b). Changes in electrophysiology at the olfactory bulb, and not the olfactory epithelium, would indicate that stimuli are being perceived at the external interface, but that this signal is not sent to the olfactory bulb for higher processing. This analysis would reveal if cadmium-metallothionein complexes in the axon terminals are interfering with sensory transduction in the olfactory bulb, leading to sensory deficits without adverse effects in the olfactory epithelium. However, these measurements may be highly technically challenging given the small size of zebrafish larvae.

Chapter 7

7. References

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