AN ABSTRACT OF THE DISSERTATION OF

Kara E Warner for the degree of Doctor of Philosophy in Toxicology presented on June 13, 2006.

Title: Environmental Estrogens and Skeletal Development Disruption in the Fathead Minnow, a Teleost Model of Vertebrate Developmental Toxicity

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Jeffrey J Jenkins

Natural and synthetic estrogenic compounds detected in surface waters have been linked to endocrine signaling disruption in several species. Endogenous estrogen contributes to cartilage and bone deposition during development in vertebrates, and is susceptible to perturbation from xenoestrogens. The goals of this work were to develop a teleost model to be used in bioassay to (a) determine the link between environmental estrogens and the morphological endpoint of vertebral abnormalities, and (b) investigate the biochemical mechanism involved in vertebral dysmorphogenesis. The hypothesis of this work is that xenobiotics with estrogenic activity adversely impact vertebral bone formation through disruption of an essential endocrine signal in bone formation, insulin-like growth factor-1 (IGF-1). Preliminary bioassay work identified the fathead minnow (*Pimephales promelas*) as an appropriate teleost model for investigating sub-lethal vertebral deformities in post-larval fish. A ranking system of relative developmental score was used to quantify the qualitative observation of skeletal development in juvenile fish. Fathead minnows were exposed to 0.1-100 µg/l 17α-ethinylestradiol (EE2) and 0.1-1000 µg/l bisphenol A (BPA) from egg stage to 25-26 days post-hatch. Fish from exposure replicates were analyzed for vertebral malformations and developmental score by fluorescently staining calcified tissues. Replicates of exposed fish were also weighed and processed to quantify IGF-1 levels in whole body homogenate. MC3T3 osteoblasts were used to characterize

in vitro bone cell proliferation and IGF-1, IGF-1 receptor, and estrogen receptor protein expression in response to EE2 and BPA exposure. EE2 and BPA treatment did not significantly affect protein levels *in vitro*, and had no significant effect on proliferation of osteoblasts in culture. However, EE2 exposure did induce an increase in vertebral deformities *in vivo* in fathead minnows, and a corresponding decrease in skeletal development. EE2-exposed fish also demonstrated a decrease in IGF-1 concentration that correlated to decreased weights. BPA did not cause significant changes in length, weight, or IGF-1 levels compared to control fish, nor did BPA-exposed fish demonstrate significant vertebral deformity. The results of these studies suggest skeletal development is a potential endpoint of endocrine disruption from potent environmental estrogens. Further IGF-1 and growth factor-related studies would provide a better mechanistic understanding of the etiology of vertebral dysmorphogenesis.

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Environmental Estrogens and Skeletal Development Disruption in the Fathead Minnow, a Teleost Model of Vertebrate Developmental Toxicity

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Kara E Warner

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Dean of the Graduate School

I understand that my dissertation will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my dissertation to any reader upon request.

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Kara E Warner, Author

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CONTRIBUTION OF AUTHORS

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TABLE OF CONTENTS

Page

Chapter 1: General Introduction

I. Endocrine Disruption, Dose-Response, and the Animal Model.....1

II. Vertebral Deformities (A Vertebrate Problem!).....8

III. Insulin-Like Growth Factor-1, Estrogens, and Bone Formation.....11

Chapter 2: Development of a Fathead Minnow Vertebral Deformity Bioassay.....22

Chapter 3: Effects of 17Alpha-Ethinylestradiol and Bisphenol A on Vertebral Development in the Fathead Minnow (*Pimephales promelas)*.....39

Chapter 4: Insulin-Like Growth Factor-1 and Impaired Development in Juvenile Fathead Minnows (*Pimephales promelas)* Exposed to 17Alpha-Ethinylestradiol.....59

Chapter 5: General Discussion.....81

References.....87

LIST OF FIGURES

Figure Page

1.1. The mechanistic basis for non-monotonic dose response.....5

1.2. Homeostatic growth factor signaling.....11

1.3. Schematic representation of IGF-1 receptor structure.....12

1.4. Fate and excretion of EE2 in the body.....16

1.5. Estrogen and IGF-1 signaling.....21

2.1. Multiple vertebral developmental abnormalities in a fathead minnow exposed to concentrated Willamette River (OR) water.....26

2.2. Fathead minnow calcified vertebrae visualized with 0.02% calcein stain.....30

2.3. Survival and deformity rates in zebrafish exposed to cadmium 14-21 days post-hatch (dph).....31

2.4. Developmental score distribution of 35 dph zebrafish exposed to (a) control (0 ug/l cadmium) and (b) 7.9 ug/l cadmium during 14-21 dph.....31

2.5. Survival and deformity in zebrafish exposed to selenium from egg stage to 7dph.....32

2.6. Survival and deformity rates in larval and juvenile fathead minnows exposed to dexamethasone at varying developmental periods.....34

LIST OF FIGURES (Continued)

Figure Page

2.7. (a) Dorsoventral curvature and incomplete calcification of spine, and (b) a compressed vertebra (indicated by arrow) in fathead minnows exposed to 100 µg/l dexamethasone at 6-9 dph (images acquired at 25+ dph).....34

3.1. 25-26 dph fathead minnow skeletons viewed by calcein stain.....48

3.2. Results for juvenile fathead minnows exposed to EE2 from egg stage to 25-26 dph.....48

3.3. Results for juvenile fathead minnows exposed to BPA from egg stage to 25-26 dph.....49

3.4. Proliferation rates for MC3T3 osteoblasts after 96-hr (a) EE2 and (b) BPA exposures.....49

4.1. Weight of fish after two 30 d exposures of 0 (solvent control) to 100 µg/l EE2 (black bars), and one 30 d exposure of 0 (solvent control) to 1000 µg/l BPA (white bars).....68

4.2. IGF-1 normalized to total protein (ng IGF-1/µg total protein) per replicate of fish after 30 d EE2 exposures.....69

4.3. IGF-1 concentration per fish (ng IGF-1/fish) after 30 d EE2 exposures.....69

4.4. Fluorescently labeled antibodies against IGF-1, IGF-1R, and ER alpha in fixed MC3T3 osteoblasts after 10 d EE2 or BPA exposure 72

LIST OF FIGURES (Continued)

Figure Page

4.5. Immunoreactive (a) ER alpha, (b) IGF-1R, and (c) IGF-1 in fixed MC3T3 osteoblast cells after 10 d EE2 (black bars) or BPA (white bars) exposure.....73

4.6. Co-localization between immunoreactive ER and Hoechst nuclear label in fixed MC3T3 osteoblast cells after 10 d EE2 (black bars) or BPA treatment (white bars).....74

LIST OF TABLES

Table Page

2.1. Exposure chemicals and concentrations used in zebrafish developmental toxicity studies.....27

2.2. Developmental scoring criteria for preliminary teleost bioassays.....28

3.1. Developmental score classification system (from Villeneuve et al. 2005).....45

Environmental Estrogens and Skeletal Development Disruption in the Fathead Minnow, a Teleost Model of Vertebrate Developmental Toxicity

Chapter 1: General Introduction

I. Endocrine Disruption, Dose-Response, and the Animal Model

More than a half-century ago, scientists were aware that DDT could affect reproductive organ development in young roosters (Burlington and Lindeman 1950). In the ensuing decades, numerous studies suggested a link between chemicals in the environment and reproductive impacts in wild bird populations (Ames 1966, Keith 1966, Wurster and Wingate 1968). Since the early 1990s, when the term 'endocrine disruption' was conceived, more than 300 papers have been published that propose a mechanism or effect of endocrine disruption.

The concept of endocrine disruption pertains to multiple chemical classes, mechanistic pathways, target organisms, and biological endpoints. In a review of the endocrine disruptor thesis, Krimsky (2001) wrote, "The term endocrine disruptor has brought attention to a class of postulated mechanisms involving hormone signal disruption that may help to explain abnormalities in humans and animals that arise at varying stages of development, in diverse physiological systems, for a number of clinical endpoints, and in response to different levels of exposure to hormonally active chemicals." Attention, indeed; rising public interest in environmental chemicals with the potential to affect reproduction, growth and development, and IQ scores increases the demands on regulatory agencies to investigate the impacts of chemicals in terrestrial and aquatic systems, in the air we breath, and in the food we eat. The task of researching potential endocrine disrupting chemicals (EDCs) and endocrine disruption is no small feat, in light of the fact that there is no globally accepted definition of EDCs; the term itself is debatable in scientific communities (Fisher 2004). The European Union and Australia use the term EDC, and while the US Environmental Protection Agency (USEPA) has adopted EDC, the US National Research Council chose the nomenclature "hormonally active agent (HAA)." Politically, the term HAA is less

pejorative than EDC; HAA denotes mechanism, while EDC suggests pathology (Krinsky 2001).

The broad concept of endocrine disruption may be narrowed to some specific conditions, as proposed by the Wingspread Conference on chemically induced alterations in wildlife and human development (Colborn and Clement 1992, Krinsky 2001). The chemicals of concern have different effects on an embryo, fetus, or perinatal organism than on an adult; the effects are most often manifested in the offspring (not the exposed parent); the timing of exposure is critical in determining future potential effects; and manifestations may not occur until maturity. A recent National Toxicology Program (NTP) review emphasizes "biologic change rather than... adverse effect because, in many cases, the long-term health consequences of altered endocrine function during development have not been fully characterized" (Melniick et al. 2002).

As the epistemological debate continues over endocrine disruption, research has begun to elucidate the molecular, cellular, organismal, and population levels effects of these compounds. Agencies within the European Union, Australia, and several multi-national coalitions have developed testing strategies to identify EDCs. The USEPA developed the Endocrine Disruptor Screening and Testing Advisory Committee (EDSTAC) in 1996 to oversee investigations into EDCs. Due to the wide-ranging chemical classes, mechanisms, and targets involved, the agency has initiated a system of high-throughput screening assays to initially identify EDCs. Subsequent, more in-depth tests in a variety of target organisms are designed to clarify mechanism. Almost a decade later, the organization is still considering appropriate testing strategies and endpoints. The NTP report cited above reviewed current literature and testing strategies in endocrine disruption research and concluded that "current testing paradigms used for assessments of reproductive and developmental toxicity should be revisited" to incorporate dose selection, age of animals at evaluation, and endpoints measured in proposed studies (Melnick et al. 2002). Additionally, studies have expanded from initial inquiries into reproductive system perturbation to thyroid hormone signaling disruption and disruption of bone mineral deposition. A recent report by Sonne et al. (2004) links organochlorine exposure – a mixture of putative estrogenic compounds – to decreased bone mineral density in polar bears of East Greenland and the Kara

Sea, areas of higher organochlorine deposition due to global atmospheric transport routes.

*****

Alongside the burgeoning EDC studies in recent decades are the advancements in chemical analytical techniques and technologies that allow researchers to identify and quantify chemicals in the environment. The EPA has identified an emerging class of compounds of environmental concern: pharmaceuticals and personal care products, or PPCPs. Of the environmental pharmaceuticals, only "a small subset of PPCPs are known or suspected of being direct-acting endocrine disrupting compounds...primarily synthetic steroids and other synthetic hormones, acting as hormone or anti-hormone modulating mimics -- agonists or antagonists, respectively" (Daughton and Jones-Lepp 2001). The low doses at which potential deleterious effects occur are of particular concern due to the wide range of concentrations at which certain chemicals may be found in the environment. Although environmental pharmaceuticals and other PPCPs may be present in very low concentrations in aquatic systems and drinking water, these compounds are often designed to have low effective doses (sub mg/kg in animals), and non-target, non-therapeutic effects can occur at very low concentrations (ug/l to ng/l) (Rodricks 2003, Daughton and Jones-Lepp 2001). Additionally, PPCPs may have undetectable or unnoticed effects on aquatic organisms if they are continually introduced to surface waters, even at low parts-per-trillion concentrations (ng/l), as their "continual infusion into the aquatic environment serves to sustain perpetual life-cycle exposures" (Daughton and Ternes 1999).

Chemicals in the EDC class may interfere with natural signaling mechanisms in an organism through hormone receptor binding and subsequent agonism or antagonism. The effect is most pronounced in developing animals below concentrations harmful to fully developed animals, and effects may not follow a traditional monotonic dose response relationship (Krinsky 2001, Melnick et al. 2002). Reports of non-monotonic, or biphasic, dose-response have existed in scientific literature since 1865, and since then have been demonstrated in multiple animal and plant models, across chemical classes and physical stressors, affecting numerous biological endpoints including homeostatic endocrine signaling (Calabrese and Baldwin 2000). The biphasic dose response

has therefore been theorized to be broadly generalizable, although it differs from both the traditional view of toxicological dose-response and the ingrained testing and interpretive strategies adopted by scientific agencies.

Testing low-dose effects (below the traditionally accepted 'no observable adverse effect level,' or NOAEL) demands powerful study designs with appropriate doses, often requiring a temporal component. Nevertheless, the NTP recommends multiple dose studies and modeling of dose-response relationships in EDC assays to incorporate low dose effects (at or below NOAELs) and non-monotonic dose-response (Melnick et al. 2002). The cellular mechanistic basis for non-monotonic dose response has been attributed in part to receptor binding, subsequent downstream signaling, and receptor expression levels in pharmacological studies. These studies indicate that for non-monotonic dose response to be detectable, a background incidence at dose 0 is a prerequisite, and that the linear default assumption (at and below NOAEL) cannot reflect the complexity of responses in a biological system. Furthermore, endocrine disruption should not be described solely by receptor-ligand interaction, but by the pleiotropic response in an organism (Connolly and Lutz 2004).

The dose response data for certain EDCs suggests the non-monotonic concept is as broadly applicable as has been suggested, and may be particularly applicable in some endocrine systems. Close to 30 receptor systems have been reported displaying biphasic dose response, including those for dopamine, testosterone, growth factors, opioids, and corticosterones (Calabrese and Baldwin 2003). Welshons et al. (2003) suggest a mechanism for the biphasic dose response stimulation of EDCs with estrogenic activity: stimulatory effects at very low doses are overwhelmed by the toxic effects at higher doses, resulting in the observed biphasic dose response (see Figure 1.1). Consideration of non-monotonic, low dose response has been integral to the hypothesis and research in this work, and care has been taken to include appropriate doses when designing experiments.

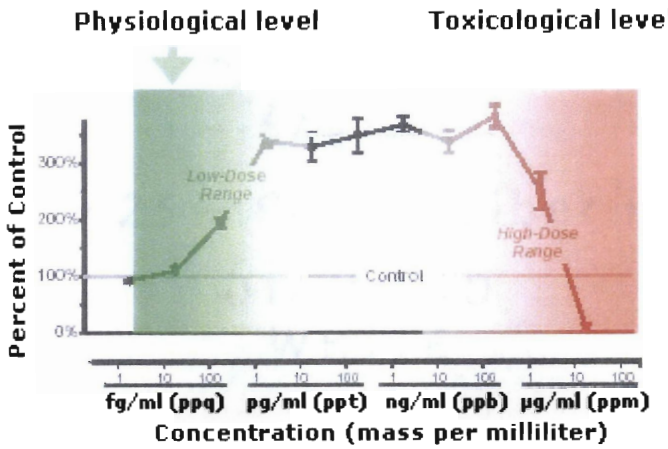

Figure 1.1. The mechanistic basis for non-monotonic dose response (from Welshons et al. 2003, reproduced with permission from *Environmental Health Perspectives*)

In addition to the concentrations of chemicals used to support the hypothesis in this work, of equal consideration were timing of chemical exposure, and selection of an appropriate animal model. As discussed, the effects of exogenous EDC exposure are paramount not only in developing organisms, but in the stage of development of that organism. Additionally, the effects of exposure may not be manifested until adulthood. One noteworthy example in human epidemiological studies is the link between diethylstilbestrol (DES) administration in pregnant women and pathological effects in the reproductive tracts of their offspring (Poskranzer and Herbst 1977). DES was shown to have no toxic effects in adults at therapeutic doses, and was widely used to prevent miscarriage in the United States from the 1940s to the 1970s. However, in up to 1.4 per 1000 exposed female offspring, vaginal and cervical cancers developed after reproductive maturity (Herbst et al. 1977). Male offspring of exposed mothers also showed a high incidence of epididymal cysts, poor semen quality, and other reproductive tract effects (Bibbo et al. 1977). The timing of exposure during fetal development was critical in DES-related pathologies; maternal use of DES prior to the 18th week of gestation was necessary for genital tract tumor formation. This example fulfills all the requirements describing endocrine disruption (Krimsky

2001), and demonstrates the impacts of an EDC on a developing organism at doses non-toxic to an adult.

The appropriate animal model for an endpoint in an endocrine disruption study is crucial, as in any toxicological study. Rats have been used to demonstrate the consistency of findings within and across animal and human species in terms of lead exposure during early neuronal development, and the neurobehavioral ramifications that occur later in life (Davis et al. 1990). Although considered a neurotoxicant, here lead is similar to endocrine disruptors in that an exposure during fetal and childhood development is critical in causing neurodevelopmental effects not seen in exposed adults (Bellinger 2004). Traditional mammalian studies are limited due to the high cost of animal care. Moreover, due to the necessity of detecting subtle effects not likely to exceed 30-60% of controls (Calabrese and Baldwin 2003), the demand for more animals to incorporate more doses becomes evident.

The teleost model therefore demonstrates a select advantage over mammalian organisms for the study of vertebral deformities: high numbers are available and maintainable at comparatively negligible cost. Marine and Freshwater Biomedical Centers (MFBSC) across the United States employ the teleost model in developmental and toxicity studies, because "as recognized by the National Institutes of Health, aquatic models can be used as sensitive, low-cost, comparative vertebrate models that reduce dependence on mammalian species for health-related research" (MFBSC, Oregon State University, www.science.oregonstate.edu/mfbsc). Unlike mammalian organisms used in research, aquatic organisms such as fish are found *in* the environment where they are directly exposed to toxic substances that may also negatively affect human health. Determination of their toxicological impact on such organisms can serve as a sentinel warning about potential adverse interaction of these chemicals in humans (MFBSC, University of Wisconsin-Milwaukee, www.uwm.edu/Dept/MFB).

As discussed, in order to establish a potential non-monotonic dose response, a low but present background level of effect must exist in the animal model at dose 0. It is desirable to select an animal model that is "reasonably susceptible to develop agent-induced disease while having a low background incidence" (Calabrese and Baldwin, 2003). The teleost fish model fulfills this critical requirement for studies with a vertebral deformity endpoint. The fathead

minnow *Pimephales promelas* is easily reared and embryos and juvenile fish are transparent, facilitating microscopy and early developmental studies. The fathead minnow has been extensively used as a teleost model in toxicological studies. Additionally, teleost fish have been used to characterize human bone disorders. The zebrafish mutant *chihuahua* has phenotypic similarities to human osteogenesis imperfecta, a skeletal dysplasia caused by mutations in the type I collagen genes, homologous to the *chihuahua* collagen I(α1) gene mutations (Fisher et al. 2003). The teleost fish is therefore a reasonable organism used to develop an animal model for human skeletal pathophysiological disorders. These factors, in combination with cost and husbandry considerations, support the fathead minnow as an ideal animal model for studying the mechanism of EDC-induced vertebral deformity. The development of the fathead minnow vertebrate model in this research, including preliminary bioassay work, is described in detail in Chapter 2.

Of final note in the consideration of endocrine disruption studies, as in any toxicity study, are the interindividual differences of organisms in response to potential EDCs and developmental toxicants. The wide ranges in response to lead (a neurotoxin) exposure in children and animal studies have been attributed to the many intervening steps that link the internal dose and the target organ (brain) response. Genetic polymorphisms in lead metabolism and coexposure to other toxicants are additional candidates for individual differences in lead toxicity (Bellinger 2004). Furthermore, environmental stimulus has been shown to directly affect hormone receptor expression. Handling of rat pups during the initial 7 days postpartum significantly increased brain glucocorticoid receptor density, increasing the brain's responsiveness to circulating glucocorticoids and reducing the stress response of the hypothalamo-pituitary-adrenal axis (Meaney et al. 1991). In cases of human idiopathic scoliosis (described in detail in section II of this introduction), the genetic background, nutritional status, and co-exposure to unknown factors may account for some individual differences in onset and severity of the disorder, but these confounding factors can be eliminated when using the teleost model. However, the response in the fish model may have substantial variability, due to the intervening steps between dose absorption, metabolism, distribution, and target organ response. To summarize, "the actions of a toxic agent in an organism [with regards to the endocrine system] are multi-

faceted, the reaction of the organism accordingly is pleiotropic, the dose response is the result of a super-imposition of all interactions that pertain" (Connolly and Lutz 2004).

The mechanism by which exogenous EDCs may induce vertebral deformities in the teleost fish is described in section III of this introduction. This mechanism conforms to the accepted concept of endocrine disruption as discussed above, and should be considered accordingly in terms of risk assessment for regulatory decision-making to protect wildlife and public health.

II. Vertebral Deformities (A Vertebrate Problem!)

> It's the least I can do since I cannot for the life of me
> think of anything but the thin curtains of a hospital room
> and an X-ray of my crooked spine pinned to a wall of light,
> the sweet milk of vertebrae, my own skull
> frowning back at me, such a cold cup of jaw,
> so white I could have easily drank myself.
>
> -Joshua Poteat "The Angels Continue Turning the Wheels of the Universe Despite Their Ugly Souls" 2004

Hippocrates, the "Father of Spine Surgery," was describing and correcting spinal curvatures in 400 B.C. (Marketos and Skiadas 1999). Galen (A.D. 130-200) termed the vertebral deformity involving lateral deviation and/or torsion of the spine "skoliosis" from the Greek word for crooked (Byrd 1988). To date, the causes of some types of scoliosis in humans have been linked to connective tissue disorders (Marfan syndrome), neuromuscular diseases (cerebral palsy), and structural deformities (hemivertebrae). The etiology of the remainder – and majority – of cases of scoliosis is not clear, however, and the condition is termed idiopathic scoliosis (Ahn et al. 2002). Idiopathic scoliosis is a condition that currently affects two to three percent of people worldwide (an estimated 6 million Americans), emerging by adolescence. Scoliosis patients suffer lateral curvature of the spine, in some cases up to 40 degrees from vertical. Complications from scoliosis involve neuromuscular pain and compression of the thoracic cavity (Szappanos et al. 1997). Each year scoliosis patients make more than 600,000

visits to private physician offices, and 38,000 people undergo spinal fusion surgery, while an estimated 30,000 children are put into a brace for scoliosis (National Scoliosis Foundation).

To date, animal models used to investigate human vertebral deformities have been limited to rodent studies involving mechanical manipulation of load asymmetry in vertebrae (Stokes et al. 1996), and mouse mutation analyses to determine a genetic component (Giampietro et al. 1999). While the former addresses the mechanical mechanism involved in progression of vertebral deformity regardless of etiology, factors influencing the onset of the deformity remains unclear. The latter study focuses on identifying human homologues to murine gene mutations, but acknowledges that idiopathic scoliosis is more likely a heterogeneous group of disorders with varied pathogenic mechanisms affecting cartilage and bone. Spinal deformities from noninfectious sources have also been reported in a number of wildlife species, including large felids (Kolmstetter et al. 2000), canids (Wobeser 1992), and birds (Hultgren et al. 1987). Aquatic vertebrates and terrestrial vertebrates closely associated with aquatic environments also demonstrate vertebral deformities. Spinal deformities have been produced in frogs due to dieldrin (a pesticide) exposure (Schuytema et al 1991), while wild sharks (cartilaginous fish) have been reported having fused or compressed vertebrae (Hoenig and Walsh 1983). Additionally, the human condition of idiopathic scoliosis has been reported in sea otters (Giddens et al. 1984, Rennie and Woodhouse 1988).

The prevalence, economic cost, and quality of life issues associated with vertebral deformities in humans and their occurrence in wildlife species leads to the necessity of developing an animal model to study the etiology and development of these types of disorders. The teleost fish model has been used in numerous developmental studies, and has proven an optimal model for toxicological studies. The incidence of skeletal deformities in teleost fish species reported worldwide supports their candidacy as a model for vertebral deformity studies. The relatively high rates of vertebral deformities (vertebral bone fusion, compression, and spinal torsions) in fish of the Newberg Pool area of the Willamette River, Oregon are of concern to scientists and surrounding community members alike (Ellis 2000). The occurrences of vertebral deformities in various fish species have been associated with nutritional deficiencies, metals such as

lead, selenium, and cadmium, parasitic infection, and with a wide range of chemical contaminants (pesticides, dioxin, pulp mill effluents). Recent studies with aquatic models have reported vertebral deformities resulting from environmental estrogens in a marine teleost (Boudreau et al. 2004, Urushitani et al. 2002) and *Xenopus* frog larvae (Iwamuro et al. 2003).

Although the mechanism of action of EDCs for reproductive endpoints has been proposed (discussed in Chapter 3), the etiology of vertebral anomalies, including scoliosis, is unclear in many reports. Animals in an aquatic system often may be exposed to a broad range of low-level contaminants, suggesting a multifactorial cause for development of skeletal deformities. Likewise, despite numerous studies that have attempted to identify a common genetic or molecular cause of idiopathic scoliosis in humans, the consensus is that the etiology of these deformities is multifactorial (Ahn et al. 2002). Factors potentially involved in this condition include genetic predisposition (Szappanos et al. 1997), anterior spinal overgrowth leading to a buckling phenomenon and worsened by bone resorption or loss (Goto et al. 2003), disruption of collagen and glycosaminoglycan synthesis (Antoniou et al. 2001), and contributions from growth-related hormones (Skogland and Miller 1980).

In an epidemiological study conducted among US adults, 8.3% demonstrated some degree of scoliosis (Carter and Haynes 1987). In the study, bone density was lower for scoliotic individuals than for non-scoliotics, suggesting an association between general bone dysmorphogenesis (including osteoporosis) and the specific morphological endpoint of scoliosis. A contributing factor in skeletal deformities may therefore involve disruption of the growth factors that signal bone patterning and deposition during development (See Figure 1.2). Disruption of homeostatic endocrine signaling by exogenous chemicals in humans and wildlife species has become extensively studied in recent decades. Growth factor perturbation may be one of the significant factors involved in the multifactorial etiology of vertebral deformities, and the hypothesis for this work concerns the disruption of bone development from a potential EDC; specifically, the deregulation of insulin-like growth factor-1 signaling by exogenous estrogenic compounds (detailed in section III of this introduction).

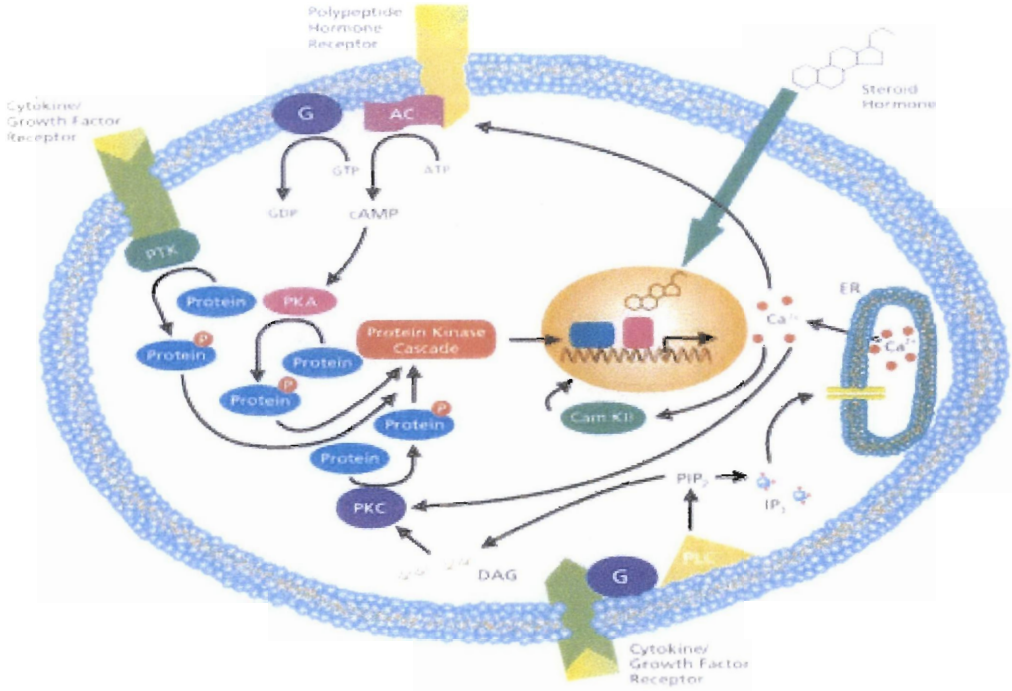

Figure 1.2. Homeostatic growth factor signaling (reproduced with permission © Sigma-Aldrich Co.)

III. Insulin-Like Growth Factor-1, Estrogens, and Bone Formation

Insulin-like growth factor 1 (IGF-1), a 7-7.5kDA peptide, has been demonstrated a critical endocrine signal for bone development in vertebrates. IGF-1 is released from the liver in response to the presence of growth hormone (GH). IGF-1 circulates throughout the body, bound by numerous insulin-like growth factor binding proteins (IGFBPs) and acts as a growth factor in numerous tissues. Upon receptor binding in chondrocytes and osteoblasts, these cells in turn release IGF-1 and IGFBPs. IGF-1 thereby serves as an anabolic hormone in bone in both a paracrine and autocrine fashion. In addition to the presence of GH, IGF-1 may be influenced by thyroid hormones (T3 and T4) in the blood, and by IGF-1 receptor (IGF-1R) expression in chondrocytes and osteoblasts. The IGF-1R is a member of the tyrosine kinase receptor family; IGF-1 thereby induces mitogenic activity through protein kinase-regulated transcription factor activation

(Adolphe 1992). A schematic representation of the IGF-1R is shown in Figure 1.3.

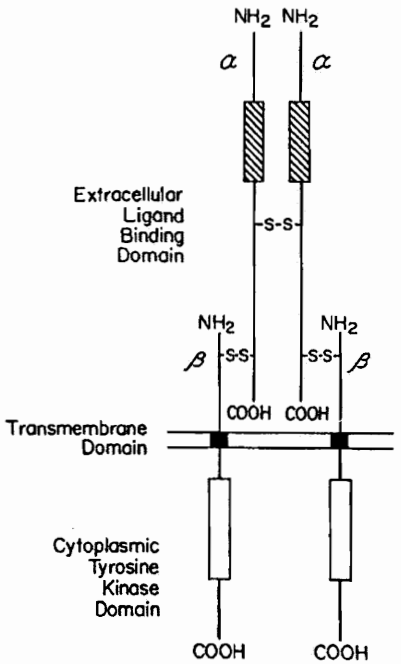

Figure 1.3. Schematic representation of IGF-1 receptor structure (© 1992 from *Biological Regulation of the Chondrocytes* by M Adolphe. Reproduced by permission of Taylor & Francis Group, LLC)

Numerous studies have been performed to demonstrate the activity of IGF-1 on developing bone tissue in mammals. In osteoblasts, IGF-1 stimulates expression of mRNA procollagen type I and subsequent collagen synthesis, and inhibits collagen degradation by inhibiting collagenase (Zofkova 2003). Additionally, IGF-1 improves the conditions for bone mineralization by acidifying the medium between osteogenic cells and the bone matrix (Sathanogopal and Dixon 1999). Neutralizing antibodies to IGF-1 and IGF-1R cause inhibition of chondrocyte proliferation *in vitro*, and cause 25 and 48 percent cell loss, respectively. Additionally, IGF-1 antisense oligonucleotides cause 28 percent loss of chondrocytes *in vitro* (Loeser and Shanker 2000). Synthetic analogues of IGF-1 designed to inhibit autophosphorylation of the IGF-1R also inhibit cell proliferation *in vitro* (Pietrzkowski et al. 1992); likewise synthetic tyrosine kinase inhibitors block the autophosphorylation and tyrosine kinase activity of the IGF-

1R, inhibiting cell proliferation (Prizas et al. 1997). IGFBPs resistant to proteolysis and therefore resistant to IGF-1 release also inhibit the activity of IGF-1 in cultured cells (Rees et al. 1998); IGFBPs serve to prolong the half-life of IGFs in the blood and at responsive tissues.

IGF-1 has also been identified in a number of fish species, and its activity has been established (Duval, et al, 2002). The relationship between IGF-1 levels and fish growth is reviewed in Chapter 4. Teleost IGF-1 and IGF-1R proteins and mRNA sequences have been cloned using cDNAs and reverse-transcriptase polymerase chain reaction (RTPCR); expression of mRNA for both has been demonstrated in embryos using *in situ* hybridization. Additionally, IGF-1R protein expression has been demonstrated in embryonic zebrafish using immunohistochemistry. Using these techniques, IGF-1 mRNA and IGF-1R have been localized in numerous tissues of adult zebrafish (Maures et al. 2002). As stated, the IGF-1Rs are members of the tyrosine kinase receptor family, and IGF-1-induced zebrafish cell proliferation is activated through the MAP kinase and PI3-kinase signaling pathways. Inhibition of these phosphorylating cascade pathways completely negates IGF-1-stimulated DNA synthesis and mitogenic actions in cultured cells (Pozios et al. 2001).

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"Estrogen" is a collective term for the sex steroids responsible for maturation of female reproductive organs and secondary sex characteristics. Extrareproductive effects of estrogen signaling in both males and females include anabolic effects on bone, stimulation of sodium reabsorption by the renal tubules, and enhanced high-density lipoprotein levels in the blood (a cardiovascular sparing effect). Estrogens (estradiol, the most abundant and efficacious endogenous estrogen, estrone, and estriol) are steroid compounds produced in vertebrate gonadal tissue as a result of follicle stimulating hormone signaling from the anterior pituitary gland, under the control of the hypothalamus. Estrogen is released from gonadal tissue and circulates throughout the bloodstream, binding to estrogen receptors (ER) in numerous tissues, including bone.

The ER is a member of the nuclear receptor superfamily, binding estrogen in the cellular cytosol compartment and migrating across the nuclear membrane

with the aid of nuclear translocase enzymes to bind to transcriptional estrogen response elements (EREs) of target genes on DNA. Membrane-bound ER may also associate with tyrosine kinase and MAP kinase protein phosphorylation cascades to increase transcriptional activity for mitogenic or apoptotic processes (Edwards 2005). Mammalian subtypes of ER include the mitogenic ERα and the anti-mitogenic ERβ. Both receptor subtypes are expressed in mammalian vertebral osteoblasts, suggesting a complex mechanism of estrogen action in bone development (Onoe et al 1997). Teleosts express three estrogen receptor subtypes, each with a different gene expression response pattern to exogenous estradiol (Menuet et al. 2004, Hawkins et al. 2000). In the Atlantic Croaker (*Micropogonias undulates*), the two forms of ERβ share amino acid changes in the ligand binding domain that may influence receptor function or ligand specificity: all three ER subtypes (ERα, ERβ, and ERβa) show specific, high affinity binding to [(3)H]-labeled 17-β estradiol, but ERβa shows higher relative binding affinity for estradiol and lower relative binding affinities for synthetic estrogens than previously characterized ERs (Hawkins and Thomas 2004). Furthermore, the 620-amino acid sequence encoding the ER [alpha] of the mummichog (*Fundulus heteroclitus*) shares 80% identity to medaka (*Oryzias latipes*) ERα, indicating a highly conserved teleost ER homology (Urushitani et al. 2003).

Estrogen has been demonstrated to play an important role in maintaining bone mass in adult humans by suppressing bone remodeling leading to bone loss and maintaining a balance between osteoblastic and osteoclastic activity. Studies in humans demonstrate the early and predominant effect of estrogen on bone remodeling: a decrease in the amount of bone resorption. Investigators have reported a variety of direct effects of estrogen on proliferation and synthesis of enzymes and bone matrix proteins in osteoblast-like cells (Oursler 1993). Estrogens have been suggested to induce ER-mediated bone growth at relatively low endogenous levels, but conversely stimulate epiphyseal fusion (inhibit longitudinal bone growth) at higher levels. In addition to their direct effect on bone, evidence exists that suggest estrogens may stimulate GH secretion from the pituitary gland, thereby increasing circulating IGF-1 (Juul 2001). Estrogen signaling may therefore impact bone formation in vertebrates in a non-monotonic pattern (see section I of this introduction).

The two endocrine signals, estrogen and IGF-1, both have significant effects on bone cell maintenance and proliferation. The question of interaction between these two growth factors subsequently arises. *In vitro* studies have elucidated the effects of estrogen on IGF-1 expression in mammalian bone cell lines. Estradiol increases gene expression of IGF-1 in rodent osteoblasts, and treatment of a human fetal osteoblast cell line with high ER levels increases steady state levels of IGF-1 mRNA in a time- and dose-dependent fashion, suggesting the IGF-1 gene is a target for estrogen action (an ERE) and that IGF-1 may mediate estrogen effects in bone (Kassem et al. 1997). Erdmann et al. (1998) reported estradiol increased the concentration of IGF-1 in bone matrix by up to 44.2% in ovariectomized rats treated *in vivo* with slightly supraphysiological estradiol (50-150nmol/kg).

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In order for exogenous estrogenic compounds to disrupt homeostatic endogenous estrogen signaling pathways, the compounds must be bioavailable to an organism, absorbed by some mechanism, distributed to estrogen-responsive tissues, and be efficacious at those tissues. Estrogen receptor presence and activity upon ligand binding have been discussed in humans and in teleost fishes above; here follows a discussion of estrogenic compounds in the environment available to aquatic organisms, and their endocrine-disrupting effects.

In a special report on pharmaceuticals and personal care products in the environment, Daughton (US EPA) and Ternes (Germany's Institute for Water Research and Technology) reviewed 66 chemicals of concern that have been measured in surface waters worldwide (1999). In their research, 17α-ethinyl estradiol (EE2), an oral contraceptive and "prime synthetic suspect regarding estrogenic effects in fish " was found at up to 7 ng/l in publicly-owned wastewater treatment plant (WWTP) effluent. More recently, Cargouët et al. (2004) published an assessment of Paris, France area river contamination by estrogenic compounds. They detected estrone, 17β-estadiol, estriol, and EE2 at concentrations ranging from 2.7 to 17.6 ng/l in WWTP effluent, and 1.0 to 3.2 ng/l in river samples. Furthermore, they reported EE2 appeared "more resistant to biodegradation in WWTPs and thus accounted for 35-50% of the estimated

estrogenic activity in rivers." A diagram describing EE2 transport to WWTP influent is shown in Figure 1.5. Putative xenoestrogens –environmental compounds exhibiting estrogenic activity – also include phytoestrogens (such as genestein from soy), detergents (such as nonylphenol), and components of plastics (such as bisphenol A). Surface water samples from China contained bisphenol A (BPA) at a range of 19-106 ng/l at 13 sites; in another site BPA was present at 8.3 µg/l (Jin et al, 2004). In a study of estrogenic compounds in WWTP effluents in southwestern Germany, estradiol was found at up to 7 ng/l, EE2 at up to 12 ng/l, the phytoestrogen genestein at up to 38 ng/l, and the estrogenic metabolites of nonylphenol polyethoxylates ranging from upper ng/l to lower µg/l range (Spengler et al. 2001).

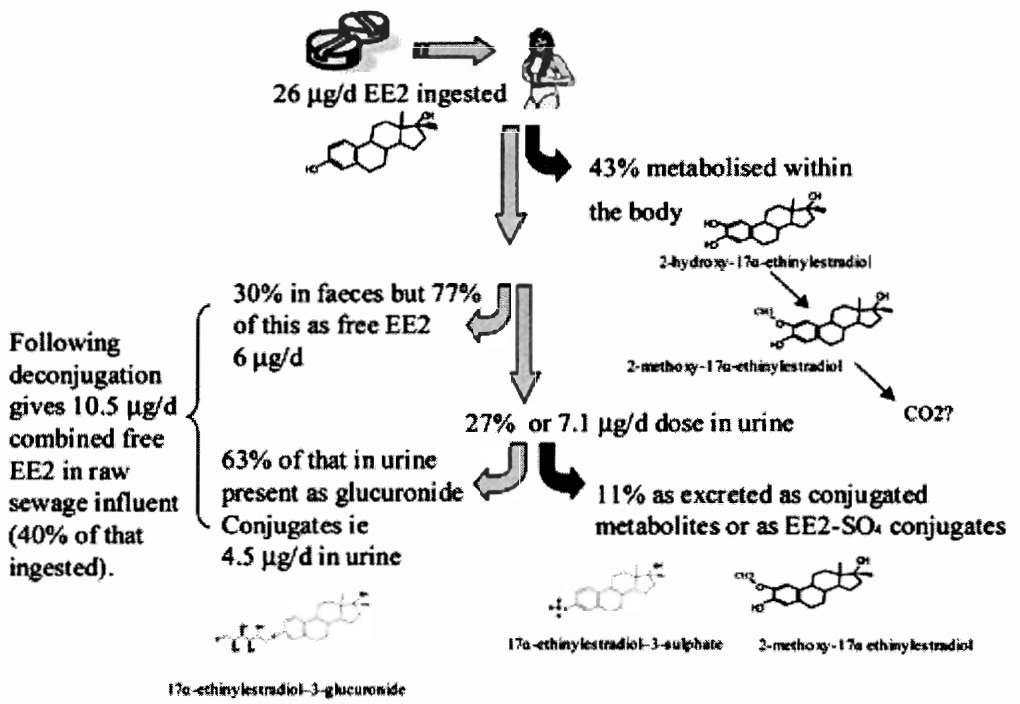

Figure 1.4. Fate and excretion of EE2 in the body (reproduced with permission from *Environmental Science and Technology* vol 38(13): 3649-3658. © 2004 American Chemical Society)

The estrogenic activity of these detected compounds has been characterized by a number of bioassays. The German group above used the E-Screen assay (a cellular proliferation assay with ER-positive human breast cancer cells) to compare the estrogenic efficacy of composite WWTP effluent (individual compounds described in Spengler et al. 2001) to the positive control 17β-estradiol. Estrogenic efficacy for the composite samples ranged from 26 to 74%; estrogenic efficacies for individual compounds were 20% for EE2, 18% for genestein, and 5% for BPA (Körner et al. 2001). In teleosts, vitellogenin levels have become a well-used biological marker for the presence of endocrine-disrupting chemicals in the environment mediated by estrogen signaling pathways; exogenous compounds may act to alter or interrupt reproductive output in fish populations. In one study (among many), laboratory *in vivo* and *in situ* field exposures to estradiol and contaminated river waters, respectively, caused significant increases in liver vitellogenin levels in rainbow trout (McClain et al. 2003).

In addition to WWTP effluent, teleosts are exposed to estrogenic compounds in surface waters from farm and feedlot waste from the over 10 million cows and 43 million swine in the United States alone. The estimated estrogen emission factor in the U.S. for 17β-estradiol is 1-10 mg/day, and 2-8 mg/day for EE2, per 1000 kg live animal weight in dairy cows. These emission rates are well over an order of magnitude above estimated estrogen mass flow from WWTPs. Furthermore, when estrogens are first excreted in both human and animal waste, they are conjugated with other molecules and are considered biologically inactive. However, microorganisms in sewage systems and waste treatment areas can break the conjugation bond, reactivating the hormones. The ethinyl group on the human synthetic estrogen EE2 blocks metabolic degradation in the environment, resulting in a half-life of several weeks (Raman et al. 2004). To demonstrate the activity of hormones in farm animal waste effluent, one research group collected wild fish and assayed for alterations in their reproductive biology. Wild fathead minnows (*Pimephales promelas)* collected from streams receiving feedlot effluent showed morphologic and endocrinologic differences from fish in streams free of feedlot discharge (Orlando et al. 2004). Responding to

increasing concern over the growth of concentrated animal feeding operations (CAFOs) and the nutrients, pharmaceuticals, and hormones present in their wastewater, the US EPA tightened regulations for CAFOs in 2003, requiring more CAFOs to seek discharge permits under the Clean Water Act (U.S.C.A. §§1251-1387), and requiring all CAFOs to develop and implement a nutrient management plan.

The effects of estrogenic endocrine disruption are not limited to reproductive endpoints. As discussed above, estrogen plays an integral role in bone deposition and maintenance, and laboratory exposures of aquatic vertebrates to environmentally relevant estrogenic compounds have resulted in vertebral anomalies. Urushitani et al. (2002) exposed mummichog (*Fundulus heteroclitus*) eggs and fry to estradiol; 85% developed crooked vertebral columns when exposed to 2.7 µg/l estradiol. Additionally, ossification was not complete in vertebrae and other bones in the treated fish; the relatively high (supraphysiological) dose may have impeded bone deposition, as described in Juul 2001. A similar study involved mummichog egg-fry exposure to EE2 for 60 days; scoliosis incidence developed in concentrations as low as 10 ng/l, and was significantly higher at 1 µg/l than incidence in controls and lower doses. Clearing and staining of larvae demonstrated expression of vertebral deformities coincided temporally with ossification (Boudreau et al. 2004). In addition to the teleost model, the *Xenopus* frog is a widely used animal in toxicological testing. High (supraphysiological) doses of estradiol and BPA have both been reported to cause vertebral deformities (crooked vertebral columns) in embryonic *Xenopus* (Iwamuro et al. 2003).

The question remains as to the levels of human exposure to estrogenic compounds. Drinking water from surface water sources is processed to remove or inactivate contaminants, so the amount of biologically active hormones in drinking water is putatively negligible. However, studies show chlorinated estradiol and halogenated nonylphenol derivatives generated during drinking water disinfection and treatment procedures demonstrate estrogenic activity, representing a potential hazard as they are able to bind ER and elicit transcriptional activation induction and proliferation (García-Reyero et al. 2004, Hu et al. 2003). Chlorinated products of BPA yield a relative binding affinity for the ER 24 times that before chlorination, and subsequent transcriptional activation

induced again suggests the chlorinated compounds "elicit the ability to mimic the effect of the estrogen hormone" (Hu et al. 2002).

BPA and nonylphenol are both widely used industrial compounds, and human exposure may come from sources other than treated surface waters. In addition to its use in food packaging, dental sealants, and manufacture of epoxy resins, BPA is a component of polycarbonate plastics, used in baby bottles and drinking bottles. E-screen assays (the human breast cancer cell proliferation assay) have been used to demonstrate significant estrogenic activity of levels of BPA (up to 310 µg/l, identified by gas chromatography/mass spectrometry) released from used polycarbonate plastic to water at room temperature; detectable levels of BPA (up to 300 ng/l) were released from new polycarbonate plastic under the same conditions (Howdeshell et al. 2003). The epoxy resin listed above is a surface coating agent on residential water storage tanks. One study reported the leaching of BPA to water from unit area of epoxy resin coating was in the range of 2-1700 µg/m^2. The amount of BPA leaching depended on the type of epoxy resin, and increased as the testing condition temperature increased, implying a higher risk of BPA contamination during warmer seasons, and a subsequent risk to human health (Bae et al. 2002). BPA used in the lacquer coating food cans in the US has been detected in water autoclaved in the cans and in the food stored in the cans at up to 80 µg/l, and the E-Screen assay was used to demonstrate estrogenic activity of the food contaminant (Brotons et al. 1995).

By whatever means humans may be exposed to estrogenic compounds, either through drinking water, foods, or domestic product use, they are consuming xenoestrogens. Calafat et al. (2005) report levels of both BPA and nonylphenol in archived human urine samples from a demographically diverse reference population of 394 U.S. adults. BPA was detected in 95% of the samples at concentrations averaging 1.33 µg/l and up to 5.18 µg/l; nonylphenol was detected in 51% of samples at up to 1.57 µg/l. According to the authors (from the Centers for Disease Control), the frequent detection suggests widespread exposure of these compounds to residents in the US.

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Based on the above findings, exposure to exogenous estrogens may therefore pose a potential threat to human and aquatic animals through disruption of homeostatic growth factor signaling. Chemicals with estrogenic activity - even lower relative binding affinity for the estrogen receptor than endogenous estradiol – may disrupt estrogen signaling pathways at low doses: the additivity of an exogenous estrogen to circulating estrogens and the subsequent lack of a threshold explain low dose effects. Exposure to a chemical acting through an endogenous mechanism may not show a threshold because the capacity of existing protective mechanisms against adverse effects has been meet or exceeded already by the endogenous hormone signals. Additivity to endogenous hormone signaling pathways is even more relevant in developing organisms: "it has been argued that one would not expect homeostatic regulatory systems that operate in adults to have a similar protective capacity in fetuses in which these very systems are undergoing development" (Sheehan, 2000).

The scientific literature reviewed here suggests a relationship among estrogenic compounds, IGF-1, and bone development. In this work, an appropriate animal model was selected and a bioassay developed to investigate the endpoint of vertebral dysmorphogenesis (Chapter 2). The fathead minnow was used to clarify impacts of estrogens on bone tissue, and to model human bone response to exogenous estrogens (Chapter 3). The hypothesis for this work is that exogenous estrogenic compounds directly and indirectly affect IGF-1 protein expression at bone tissue, and induce vertebral deformities in exposed animals through the disruption of homeostatic IGF-1 signaling (Chapter 4). A diagram of the role of xenoestrogens in endogenous IGF-1 signaling is depicted in Figure 1.6. Teleost and human vertebral deformities are sublethal conditions of generally idiopathic etiology, and this work investigates a potential mechanism of bone growth signaling perturbation by exogenous EDCs.

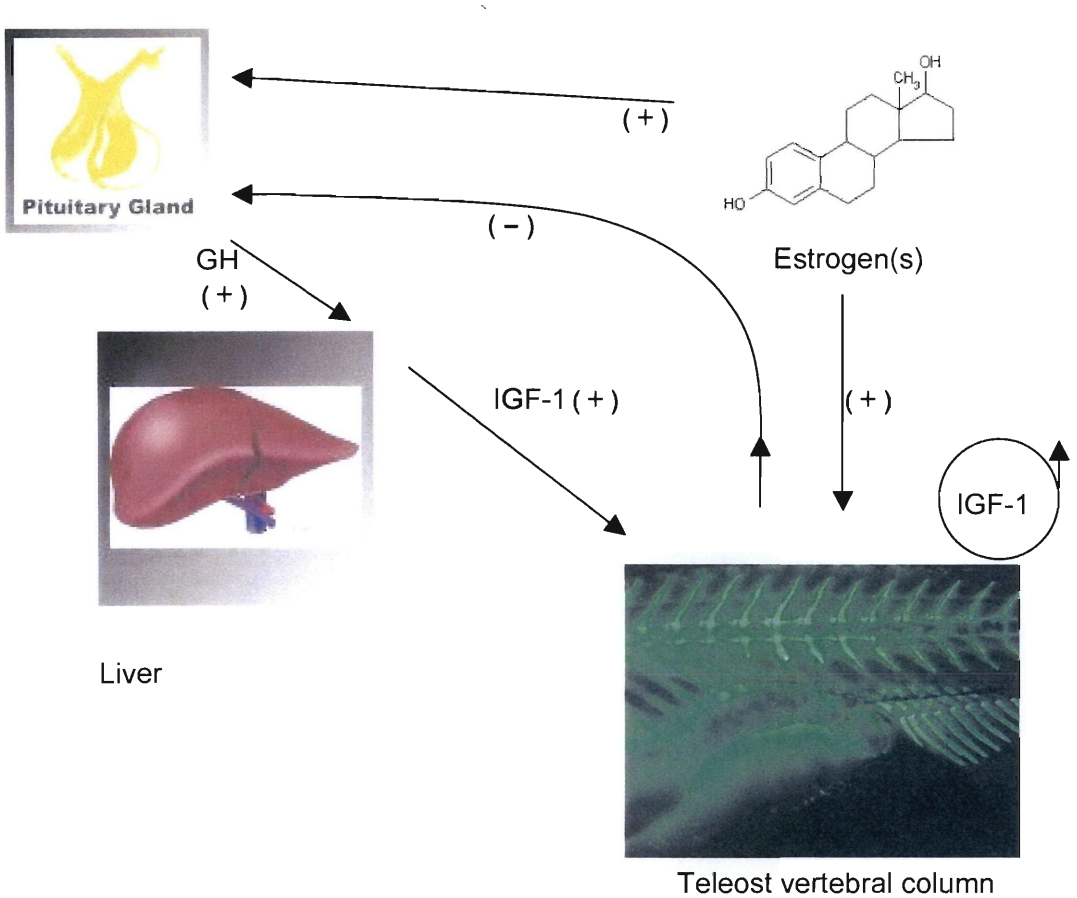


Figure 1.5. Estrogen and IGF-1 signaling

Chapter 2: Development of a Teleost Vertebral Deformity Bioassay

Abstract

High rates of skeletal anomalies including fused vertebrae and spinal curvatures have been reported in several species of fish collected from the Willamette River, OR. Bioassay is a useful tool in developmental toxicity studies to determine effects of potential chemicals of concern. This report describes the development of a teleost bioassay used to investigate the effects of test chemicals with reported vertebral teratogenic potential, and provide a positive control for experimental compounds. Embryo-larval zebrafish (*Danio rerio*) were exposed 0.4, 2.0, or 7.9 µg/l cadmium, 1-10 mg/l selenium, or 0.064-200 µg/l chlorpyrifos in a duration of 6-7 days starting at either 1 day or 14 days post-fertilization (dpf). Fish were examined on either 7 or 28-35 dpf for gross abnormalities using light microscopy or fluorescence microscopy and a fluorescein stain to illuminate calcified tissue, respectively. Zebrafish exposed to cadmium 14-21 dpf and to selenium 1-7 dpf demonstrated a statistically nonsignificant increase in morphological abnormalities. There was no concentration-dependent increase in skeletal deformities in zebrafish exposed to chlorpyrifos 14-21 dpf. Overall, the zebrafish model is not well suited for providing the number of fish needed for this type of screening assay due to high, variable mortality observed. A second model teleost, the fathead minnow (*Pimephales promelas*), was used in bioassays targeting potential windows of vertebral development susceptible to endocrine-mediated growth factor signaling disruption in bone. The rapid skeletal development and hardy nature of the fathead minnow make them a well-suited model for determining exogenously induced endocrine disruption in developing vertebrae of post-larval fish. Juvenile fish were exposed to 0.01-10000 µg/l dexamethasone for 96 h during varying stages of vertebral development (2-5, 6-9, and 10-13 days post-hatch (dph)). Fish were analyzed microscopically for vertebral malformations at 25+ dph using fluorescein stain and fluorescence microscopy. Deformities ranged from vertebral compression and bone fusion to severe scoliotic curvatures; fish exposed to 100 µg/l dexamethasone developed the most deformities (up to 17% affected fish, a 3-fold increase over controls). The results of these investigations suggest the fathead minnow may be a useful model organism in bioassays to identify contaminants capable of inducing sub-lethal skeletal deformities.

Introduction

The Willamette Valley in western Oregon is home to 70% of the state's 3.5 million residents, and is comprised of both urban areas and one of the most highly productive agricultural areas in the Pacific Northwest (Wentz et al. 1998). The Willamette River flows through the valley over 180 miles before joining the Columbia River, and receives more runoff per square mile of water shed than any other U.S. river (Urich and Wentz 1999). In 2001 the Oregon Watershed Enhancement Board funded a cooperative project at Oregon State University to investigate the cause(s) of skeletal deformities reported in Willamette River fish, including possible links between the abnormalities and chemicals from anthropogenic sources. Historical studies documented deformities in caudal, fin, and skull skeletal tissues of Willamette River fishes, but the majority (87-100%, depending on species) of deformities consisted of spinal anomalies such as lordosis (dorsoventral curvature of the spine) and fused vertebrae, affecting up to 74% of fish in some areas of the river (Ellis 2000). More recently, Markle et al. (2002) reported similar deformities in 25% of fish in ten species collected from the Newberg Pool area (river mile 55 to 26.5) of the Willamette River.

Vertebral deformities in fish as markers of chemical exposure have been reported in scientific literature for over 60 years. A wide variety of chemical and biological agents induce deformities through a host of different mechanisms (reviewed by Villeneuve et al. 2005). The OWEB-funded project at Oregon State University was a collaborative effort among the departments of Fisheries and Wildlife (Doug Markle), Environmental and Molecular Toxicology (Larry Curtis, Jeff Jenkins, and Kim Anderson) and Microbiology (Mike Kent) to assess the current prevalence of fish deformities in Willamette River areas, characterize the distribution of selected chemical pollutants in those areas, and perform laboratory experiments to investigate the ability of selected stressors to induce vertebral deformities in a bioassay. The data for this chapter were generated in part by the group performing toxicity studies on bioassay-directed river water fractions (Larry Curtis, Jeff Jenkins, Dan Villeneueve, Fred Tilton, and Kara Warner). A summary of the results of the river water bioassays (and results from the cooperative studies) was previously reported in Villeneuve et al. (2005).

This chapter focuses on the development of the teleost bioassay and the endpoint of vertebral deformities in laboratory fish models similar to those

reported in Willamette River fishes (see Figure 2.1). Three "positive control" compounds were investigated with known or proposed mechanisms of vertebral teratogenic action on the vertebral deformity endpoint. In the positive control bioassays, we tested the ability of two metals and an organophosphate pesticide reported in scientific literature to induce vertebral dysmorphogenesis in fish. Cadmium impairs myotome formation in zebrafish embryos (Cheng et al. 2000); selenium perturbs protein synthesis (Teh et al. 2002). Chlorpyrifos decreases the compressive strength of vertebrae, and may inhibit collagen formation (Karen et al. 2001). For this investigation we exposed zebrafish (*Danio rerio*) to chemicals at late embryonic stages, and early and late fry stages to correlate chemical exposure to the developmental effect of vertebral deformity. Zebrafish were chosen as a model organism due to their relatively rapid developmental rate (egg to full axial skeleton in 30 days) and their fecundity.

Endocrine disrupting chemicals are a class of potential environmental toxicants in aquatic systems, and recent reports focus on the relative dose dependency of exogenously induced hormone signaling effects, and the mechanistic basis through which putative endocrine disruptors act. In 1999 the U.S. Environmental Protection Agency (USEPA) designated these compounds of emerging environmental concern the classification "Pharmaceuticals and Personal Care Products," or PPCPs. Of the environmental pharmaceuticals, only "a small subset of PPCPs are known or suspected of being direct-acting endocrine disrupting compounds...primarily synthetic steroids and other synthetic hormones, acting as hormone or anti-hormone modulating mimics -- agonists or antagonists, respectively" (Daughton and Jones-Lepp 2001).

Following completion of the Willamette River study, subsequent bioassays were therefore conducted testing the ability of a pharmaceutical agent, dexamethasone, to induce vertebral deformities similar to those elicited in the positive control bioassays discussed above. Synthetic glucocorticoids like dexamethasone are widely used pharmaceutical agents in both human and veterinary medicine as potent anti-inflammatory drugs. Oral corticoids are the 8th most prescribed class of drugs, with an estimated 37 million prescriptions in 2002 in the U.S. (Daughton and Jones-Lepp 2001). Both metabolites and up to 60% of unmetabolized parent drug are excreted in the urine, and may therefore be accessible to aquatic organisms (Diedrich et al. 1998, Miyabo et al. 1981).

Dexamethasone has been shown to stimulate osteogenic differentiation and induce insulin-like growth factor -1 (IGF-1) binding protein expression (reviewed in Chapter 1) preferentially in vertebral bone (Milne et al. 1998a, 2001). Synthetic corticosteroids and other compounds with corticoid-like activity may induce vertebral chondrocyte proliferation through direct action on bone tissue, and indirectly through activation of thyroid-stimulating hormone. Thyroid hormone (T3) markedly increases IGF-1 expression in vertebral cell cultures (Milne et al. 1998b), and exogenous corticosteroids induce á rapid increase of circulating IGF-1 in euthyroid individuals (Prummel et al. 1996).

The teleost model for potential endocrine disruption in the glucocorticoid study was the fathead minnow (*Pimephales promelas*). Fathead minnows are easily reared in the laboratory, and have been demonstrated a useful model for toxicity studies. The vertebrae of fathead minnows develop more quickly than zebrafish, both members of the Cyprinid fish family. Cyprinid minnows are reported as the most prevalent fish with spinal deformities in the Willamette River (Markle et al. 2002).

Both zebrafish and fathead minnow bioassays were designed to investigate potential windows of early development wherein exposed fish may be susceptible to perturbation of bone formation processes. Bioassays involved semi-static to daily static renewal, and exposure routes of chemicals included gill and dermal surfaces and digestive routes, as food(s) were added to the exposure solutions with the fish. At the end of exposure or rearing, fish were analyzed microscopically for vertebral deformities including scoliosis (lateral curvature), dorsoventral curvature, and compressed or fused vertebrae. These types of deformities have been reported in fish species in oceans, lakes, and rivers worldwide, including the Willamette River, and concern scientists, aquaculturists, and surrounding community members alike. Mortality and developmental score (a comparative ranking of skeletal development of juvenile fish) were also recorded as endpoints in some cases. The bioassay developed here may prove useful in future putative endocrine disruptor studies to elucidate risk of bone dysmorphogenesis in wildlife and humans from environmental chemical exposure.

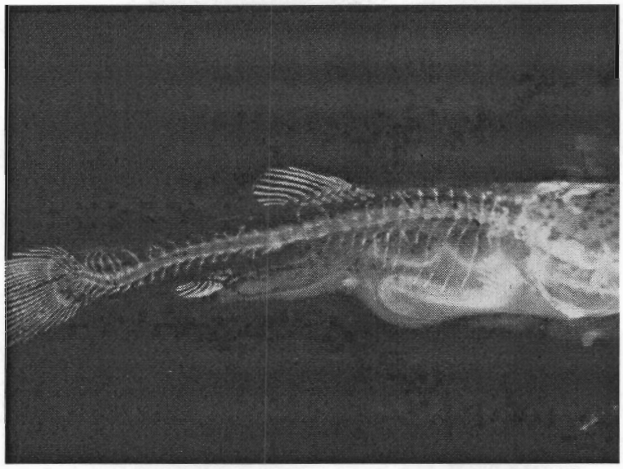


Figure 2.1. Multiple vertebral developmental abnormalities in a fathead minnow exposed to concentrated Willamette River (OR) water

Materials and Methods

Zebrafish Bioassays

Zebrafish eggs were obtained by breeding adult Tuebingen Line (a wild-type line developed in Germany) fish housed at facilities at Oregon State University. Eggs were collected three to seven hours post-fertilization.

Two exposure windows were selected: (1) embryo-larval exposure 1-7 days post-fertilization (dpf), and (2) a time point later in development (14-21 dpf) during vertebral calcification and development. Fish were exposed to cadmium (48-hr static renewal), selenium (48-hr static renewal), or chlorpyrifos (24-hr static renewal) with appropriate controls; concentrations are in listed in Table 2.1 (unless otherwise noted, reagents were obtained from VWR, Westchester, PA, USA). Exposure conditions were in 1 l glass jars; total volume of solutions was 0.5 l. The number of fish/eggs per container (15-50) and the number of replicates per treatment (3-6) varied, depending on the experiment. Duration of exposure was 7 days, with a temperature of 27±1 ºC and a 16/8 hr light/dark photoperiod cycle. Food consisted of paramecia cultured in the lab (Zebrafish International Resource Center, Eugene, OR, USA) fed twice daily at 5-20 dpf and brine shrimp cultured in the lab (Brine Shrimp Direct, Ogden, UT, USA) fed twice daily at 12-35 dpf.

In the embryo-larval exposures, 7 dpf fry were anaesthetized with 200 mg/l tricaine methane sulphonate (MS-222, Finquel, Redmond, WA, USA) and examined using light microscopy on a Leica MZFL111 dissecting microscope

(Bartles and Stout, Bellevue, WA). For more developed fish, 28+ dpf fry were immersed in a 0.02% calcein (Sigma-Aldrich, St. Louis, MO, USA) solution for ten minutes, then in fresh water for ten minutes, and anaesthetized with 200 mg/l MS-222 and examined on the Leica dissecting microscope with a fluorescein filter. Images were acquired using ImagePro Plus (Media Cybernetics, Silver Springs, MD).

Fish were counted to assess mortality, and examined for general developmental abnormalities, specifically vertebral malformations including twisting, scoliosis, and fused or compressed vertebrae. In some cases fish were also examined for vertebral skeletal growth, and given a developmental score describing comparative skeletal development (see Table 2.2). Healthy 35 dph zebrafish possess a fully ossified vertebral column, with neural and haemal processes projecting from each vertebra. Disruption of full growth potential at this stage of development includes a reduction in vertebral process number and size, or more severely a reduction in the number of calcified vertebrae.

Survival and vertebral deformity for each experiment were analyzed by one-way analysis of variance (ANOVA) with Tukey-Kramer post-tests.

Table 2.1. Exposure chemicals and concentrations used in zebrafish developmental toxicity studies

Test Chemicals	Concentrations	Carrier Solvent	Exposure Window
cadmium	0, 0.4, 2, 7.9 µg/l*	n/a	14-21 dpf
selenium	0, 1, 2.5, 5,10 mg/l**	n/a	1-7 dpf
chlorpyrifos	0, 0.064, 0.32, 1.6, 4, 8, 40, 200 µg/l**	acetone (0.01%)	14-21 dpf

*Actual concentration
**Nominal concentration

Table 2.2. Developmental scoring criteria for preliminary teleost bioassays

Developmental Score	Description
1	Vertebral ossification anterior to pelvic fins only. Little or no fin ray development.
2	Incomplete spinal development. Vertebral development extends past pelvic fin region but is not complete all the way to caudaul fin. Moderate fin ray development.
3	Vertebrae fully developed from head to caudal fin. Not all vertebrae have visible, paired neural and haemal spines. Ossified ribs not visible. Incomplete fin ray development.
4	Vertebrae fully developed from head to caudal fin. Neural and haemal spines visible and present on all vertebrae. Ossified ribs visible. Moderate fin development.
5	Fully developed skeletal structure from head to caudal fin. Fully developed complement of fin rays evident.

Fathead Minnow Bioassays

Fathead minnows (*Pimephales promelas*) were acquired from Chesapeake Cultures (Hayes, VA, USA) at select stages of post-hatch development. Fish were acclimated for no less than 3 hours to exposure temperature (22-23 °C) before chemical exposure.

Exposure periods were selected to capture stages of increasing vertebral development and ossification. Fathead minnows develop relatively quickly, and vertebrae are completely ossified typically by day 15 post-hatch (Figure 2.2). Exposure windows were 2-5 days post hatch (dph), 6-9 dph, and 10-13 dph. After acclimation, 30 fish were randomly transferred by pipet to exposure beakers. Three replicate beakers of each concentration were prepared for each experiment.

Dexamethasone solutions were prepared using serial dilutions from a 1 mM (10^{-3}M, 10 mg/l)) solution (with ethanol solvent) prepared fresh at the beginning of each exposure. Dilutions were prepared to yield 10^{-5} M and 10^{-7} M solutions. 2ml of each solution were added to each appropriate beaker, and dechlorinated filtered water was added to bring the total volume to 200 ml,

resulting in final solutions of 10^{-9} M, 10^{-7} M, and 10^{-5} M (or 0.01 µg/l, 1 µg/l, and 100 µg/l, respectively). Final ethanol solvent load for each concentration (including solvent control) was 0.1%.

Exposures were performed in 400 ml glass beakers at 22-23ºC, aerating throughout the exposures. Fish were fed spirulina algae (Algae Feast, Earthrise, Petaluma, CA) at 2-4 dph, and brine shrimp (GSL Brine Shrimp, Ogden, UT) twice daily at 5-13 dph. Water quality parameters (pH, nitrate, and ammonia levels) were recorded daily (dissolved oxygen was not recorded due to continuous aeration during exposures).

Daily renewals of dexamethasone solutions were performed during the exposures. Then, after several renewals of fresh dechlorinated filtered water to remove chemical, fish were transferred by pipet to 1 l polyethylene containers and moved to a flow-through system of dechlorinated filtered water for rearing until 25+ dph. Water quality was checked weekly and fish were fed brine shrimp twice daily; spirulina algae was added once weekly to supplement the brine shrimp diet. During the exposure and grow-out period, fish were maintained in a 16 hr light/8 hr dark photoperiod.

On or after 25 dph, fish were immersed in calcein stain and analyzed as described in the above section. Fish were counted to assess mortality, and examined for developmental score (Table 2.2) and vertebral malformations, including twisting, scoliosis, and fused or compressed vertebrae.

Survival, developmental score, and vertebral deformity for each experiment were analyzed by one-way analysis of variance (ANOVA) with Tukey-Kramer post-tests.

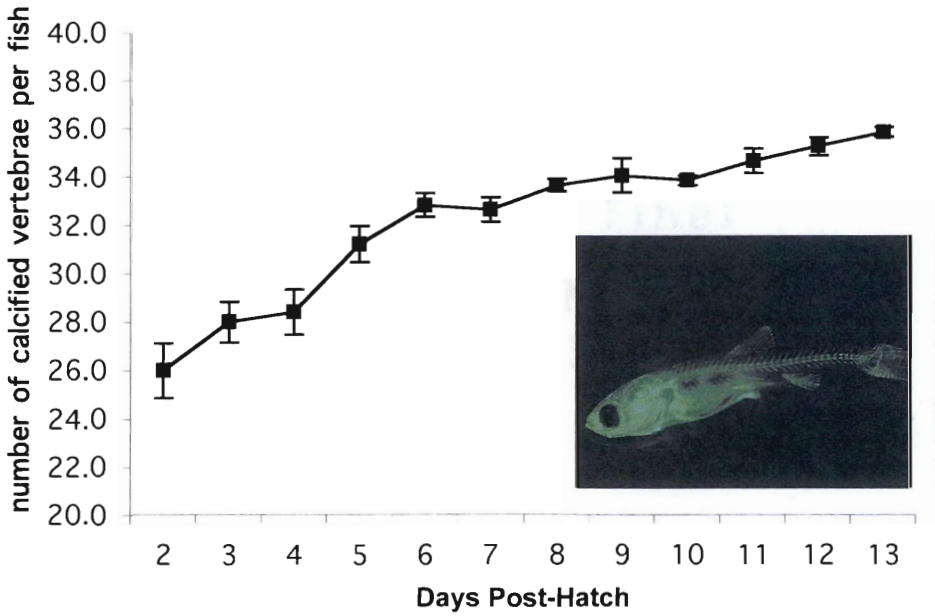


Fig 2.2. Fathead minnow calcified vertebrae visualized with 0.02% calcein stain. Five developing fish were analyzed daily from 2-13 dph. By 25 dph (insert), vertebrae have developed processes to support musculature.

Results

Zebrafish Bioassays

Skeletal abnormalities were observed in 8-15% of zebrafish exposed to 0.4, 2, or 7.9 µg/l cadmium from 14-21 dpf. However, this was not different from the deformity rate of controls (9.4%). Long-term survival in cadmium trials was highly variable. Survival at the end of exposure (2 dpf) was ≥50% in all treatments; survival at the end of the experiment (35 dpf) was ≤40% for all treatments (see Figure 2.3). Developmental score of cadmium-exposed zebrafish was affected in the highest cadmium concentration tested (see Figure 2.4).

Zebrafish larvae exposed to 1-10 mg/l selenium at 1-7dpf showed a nonsignificant increase in morphological abnormalities at 2.5 and 5 mg/l selenium exposure (Figure 2.5). Survival at the end of exposure (and end of experiment, 7 dpf) in three trials with selenium was ≥60% in all treatments except for 0% at 10 mg/l. Zebrafish larvae also suffered a significant concentration-dependent increase in cardiac or abdominal edema ($p \leq 0.03$, data not shown), which may have affected survival at the highest concentration.

There was no significant concentration-dependent increase in skeletal deformities in zebrafish exposed to 0.064-200 µg/l chlorpyrifos from 14-21 dpf. Survival at the end of exposure (21 dpf) in controls was 75%, and highly variable in all treatments at the end of exposure and at end of the experiment (35 dpf). No fish survived to 35 dpf after 200 µg/l chlorpyrifos exposure at 14-21dph (data not shown).

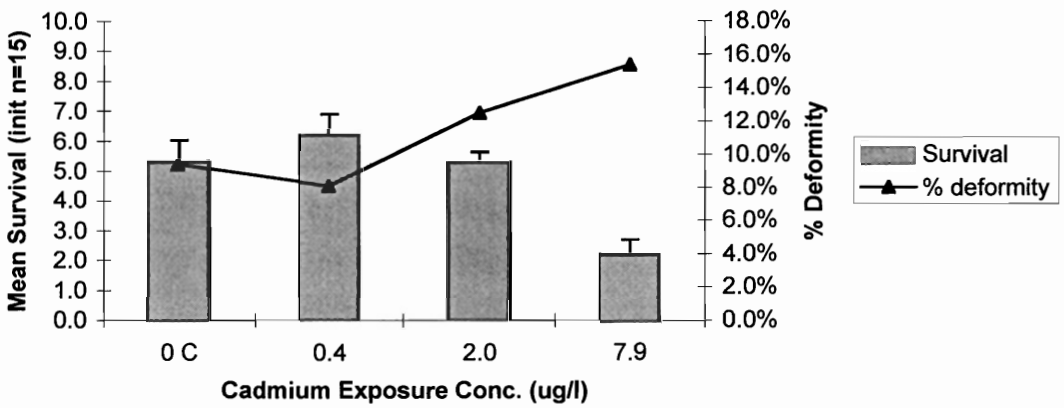


Fig 2.3. Survival and deformity rates in zebrafish exposed to cadmium 14-21 days post-hatch (dph). Survival and deformities were assessed at 35 dph. Percent deformity represents number of deformed fish divided by total surviving fish from 6 pooled replicates for each concentration.

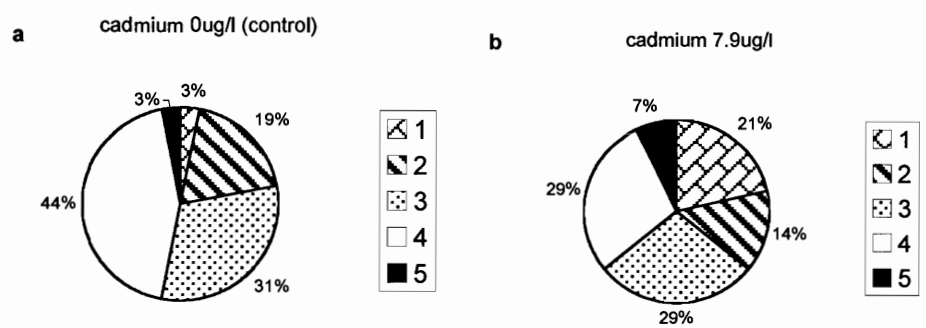


Fig 2.4. Developmental score distribution of 35 dph zebrafish exposed to (a) control (0 ug/l cadmium) and (b) 7.9 ug/l cadmium during 14-21 dph.

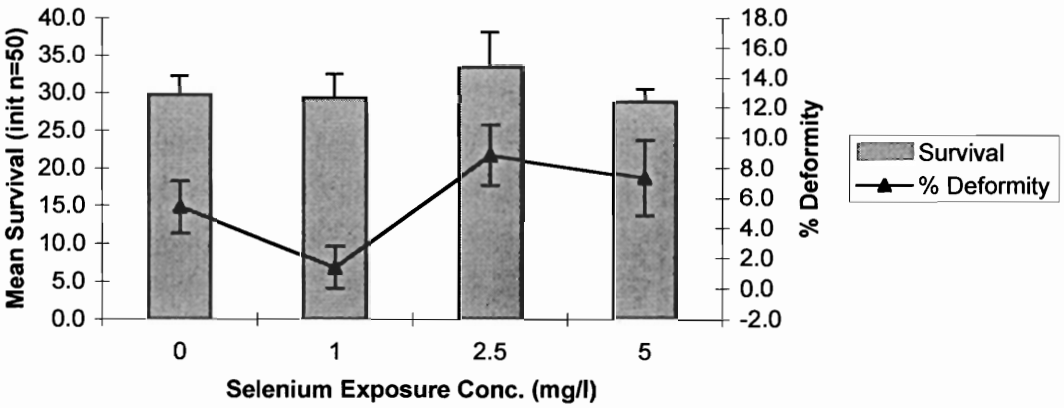


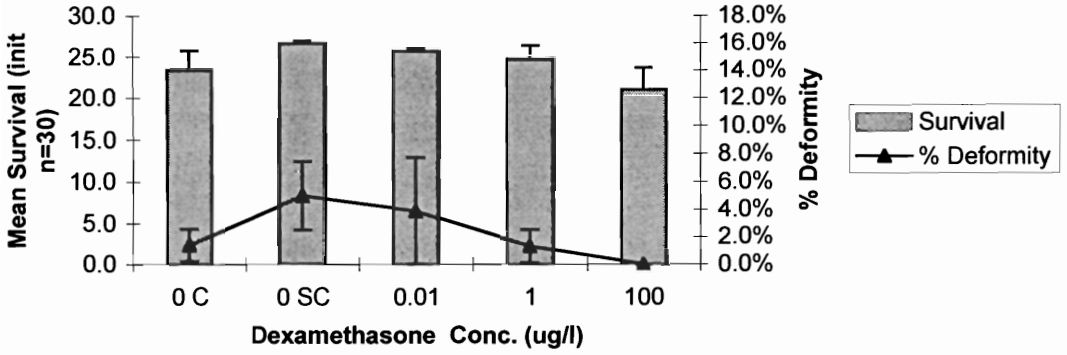
Fig 2.5. Survival and deformity in zebrafish exposed to selenium from egg stage to 7dph; survival and deformities were assessed at 7dph. % Deformity represents number of deformed fish divided by surviving fish from each of 3 replicates for each concentration.

Fathead minnow bioassays

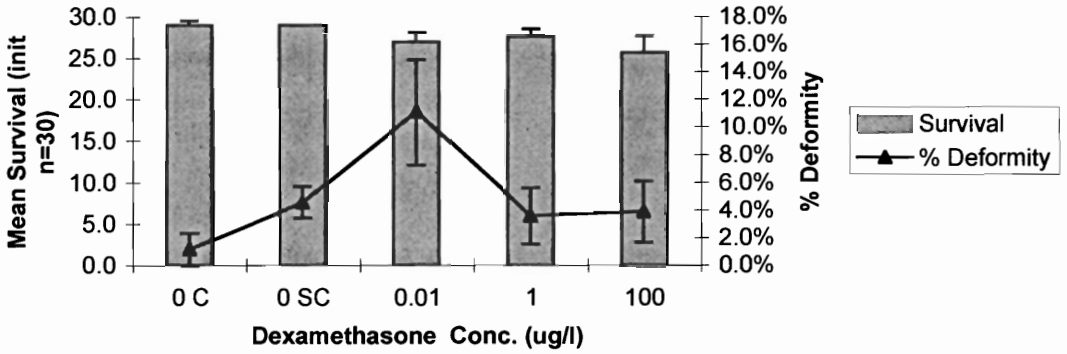
Survival to 25+ dph was ≥80% for juvenile fathead minnows exposed to dexamethasone at each stage of development, with the exception of a 10^{-3} M concentration (10 mg/l) at 10-13 dph, wherein all exposed fish died. Developmental scores of dexamethasone treated fish did not significantly differ from those of control fish (data not shown).

Deformity rates were variable in all experiments performed. Deformity rates of dexamethasone exposed fish did not significantly differ from controls. The largest response (14% deformed fish) occurred at 100 µg/l (Figure 2.6). Deformities included scoliosis and other curvatures, vertebral bone compression, and bone fusion (Figure 2.7). Deformities also included extra, missing, or deformed haemal and neural spinous processes. Deformity type was not related to exposure concentration.

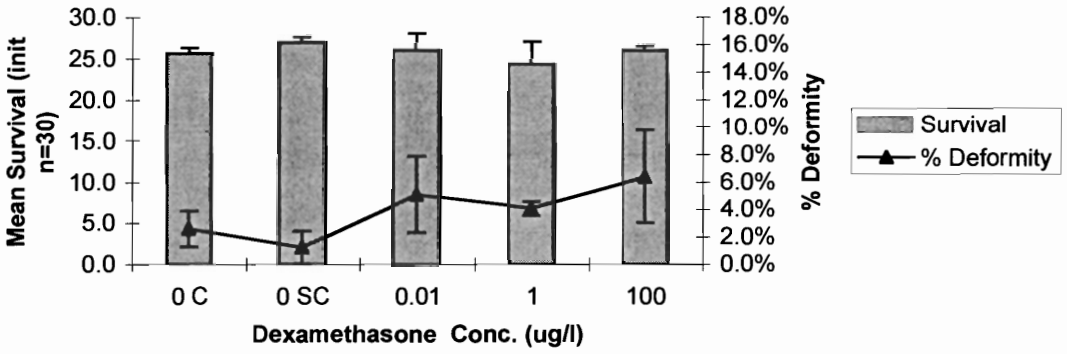
(a): 2-5dph



(b): 6-9dph



(c): 10-13dph



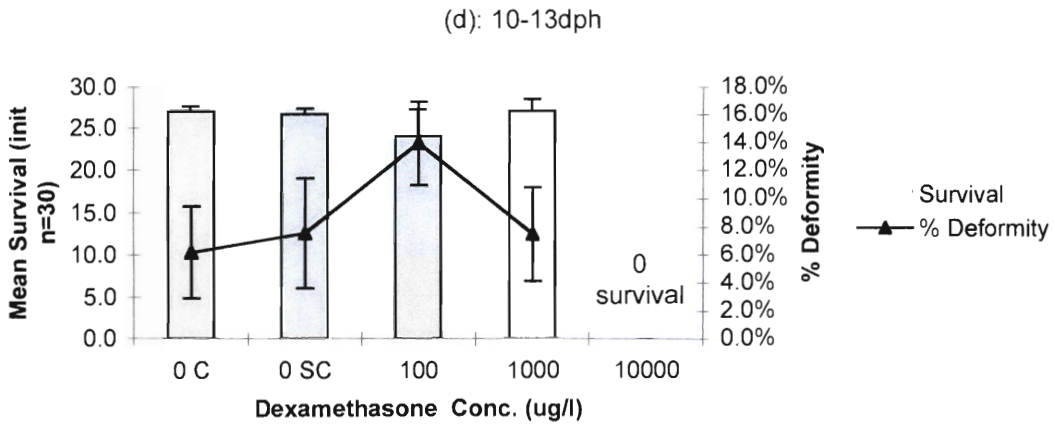


Fig 2.6. Survival and deformity rates in larval and juvenile fathead minnows exposed to dexamethasone at varying developmental periods: (a) 2-5 dph, (b) 6-9 dph, (c) 10-13 dph, and (d) 10-13 dph at higher dexamethasone concentrations. Survival and deformities were assessed at 25+ dph. Percent deformity represents number of deformed fish divided by surviving fish from each of 3 replicates for each concentration.

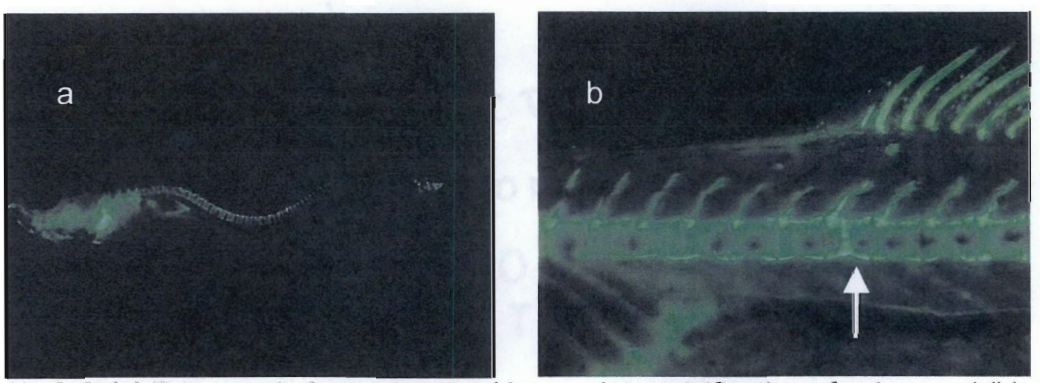


Fig 2.7. (a) Dorsoventral curvature and incomplete calcification of spine, and (b) a compressed vertebra (indicated by arrow) in fathead minnows exposed to 100 µg/l dexamethasone at 6-9 dph (images acquired at 25+ dph).

Discussion

Skeletal deformities were observed, though often with high variability, for each chemical tested in this study. The highest deformity rates produced in these studies were 16% (selenium) and 14% (dexamethasone). Rates of deformed fish in areas of the Willamette River considered reference areas (upstream of major agricultural and municipal areas) are ≤5%, similar to the "background" rates of deformities in control fish in our experiments.

Low and variable survival in treated and untreated zebrafish confounded results. Embryonic zebrafish treated 1-7 dpf with slight to severe deformities rarely survived beyond 10 dpf, reducing our ability to demonstrate long-term effects of early chemical exposure. These data represent an effort to demonstrate the effectiveness of zebrafish as a model organism in short-term bioassay studies of sublethal chemical exposure. Despite their extensive use in similar toxic screening bioassays as embryos, zebrafish may be less suited to a juvenile skeletal deformity endpoint.

We used fathead minnows (*Pimephales promelas*) as a second model teleost in the glucocorticoid exposure bioassays, with considerably better survival and endpoint results. Based on these bioassays, the fathead minnow is a more suitable teleost model for sublethal vertebral dysmorphogenesis studies in juvenile fish. The fathead minnow has a faster development from egg to fry than the zebrafish, and has also been used as a model organism in toxicity bioassays. Results from the glucocorticoid exposure study suggest a non-monotonic dose response in fathead minnows to increasing concentrations of dexamethasone exposure. The highest deformity rate was observed in 100 ug/l, after which deformity rate decreased, while toxicity increased (as represented by 0% survival of fish in the highest concentration tested). These data conform to biphasic responses seen in some endocrine disruption studies (Calabrese and Baldwin 2003).

Despite the limited ability of the selected compounds in these experiments to induce significant vertebral deformities, several benefits did arise from this research. The bioassays identified the fathead minnow as an appropriate teleost model for studies involving post-larval life stages. Additionally, criteria used in a comparative skeletal development ranking system were established and published in a peer-reviewed journal to aid researchers in quantification of a qualitative morphological endpoint of chemical exposure. Results from the glucocorticoid studies suggest a longer window of exposure may be necessary to induce endocrine disruptor-mediated vertebral deformities; future studies will be designed to include chemical exposures throughout the duration of vertebral bone development. Future experiments will also include *in vitro* assays to test the mechanistic hypothesis of endocrine disruption in developing bone from environmental chemical exposure.

The EPA has suggested pharmaceutical and personal care product compounds are potential sources of concern due to their ubiquitous use in human and animal populations (Daughton,and Jones-Lepp 2001). The concentration range for glucocorticoids in surface waters is unknown, prohibiting studies to investigate the risk of vertebral dysmorphogenesis from environmentally relevant levels of chemical exposure. However, the concentrations of estrogen and estrogenic compounds in surface waters from around the world have been documented (reviewed by Daughton and Ternes 1999). Endogenous estrogen plays a role in regulating bone growth and remodeling (Oursler et al. 1993), and exogenous estrogens may therefore disrupt endogenous endocrine control of bone formation processes. Future experiments to elucidate disruption of homeostatic endocrine control of vertebral bone formation will include bioassays optimized in this research, with environmentally relevant concentrations of estrogenic compounds, and biochemical assays to determine endpoints in the estrogen and IGF-1 signaling pathways susceptible to xenoestrogens.

Acknowledgments

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EFFECTS OF 17ALPHA-ETHINYLESTRADIOL AND BISPHENOL A ON VERTEBRAL DEVELOPMENT IN THE FATHEAD MINNOW (*PIMEPHALES PROMELAS*)

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Effects of 17Alpha-Ethinylestradiol and Bisphenol A on Vertebral Development in the Fathead Minnow (*Pimephales promelas*)

Abstract

Growth, reproductive ability, and metabolic functions may be impaired by disruption of early endocrine patterning. Natural and synthetic estrogens detected in surface waters have been linked to endocrine signaling disruption in several species. This study characterized vertebral anomalies as a morphological endpoint of endocrine disruption in fish exposed to environmental estrogens. Estrogen is a proliferation-inducing compound in osteoblasts, regulating cartilage and bone deposition during development in vertebrates. The hypothesis for this work is that xenobiotics with estrogenic activity adversely impact vertebral bone formation. Fathead minnows (*Pimephales promelas*) were exposed to 0.1-100 µg/l 17α-ethinylestradiol (EE2) and 0.1-1000 µg/l bisphenol A (BPA) from egg stage to 25-26 days post-hatch. Fish were measured for length and analyzed microscopically to determine developmental score and the occurrence of spinal abnormalities including vertebral compression, bone fusion, and spinal curvatures. In addition to the *in vivo* assays, MC3T3 osteoblasts were used to characterize *in vitro* bone cell proliferation responses to EE2 and BPA. Vertebral malformations were observed in up to 62% of EE2 exposed fish in a concentration-dependent manner; BPA induced similar malformations at only the highest concentration tested in 15% of fish. Skeletal developmental was significantly affected in EE2 exposed fish, and was inversely related to vertebral malformation rates in both EE2 and BPA treated fish. The bioassay results suggest homeostatic endocrine signaling during vertebral bone development is a potential endpoint of endocrine disruption from potent estrogenic compounds in surface waters.

Introduction

Alterations in homeostatic endocrine function in early development of an organism may have far-reaching implications in many physiological processes later in life. The protective mechanisms against chemical insult that operate in adults are themselves undergoing development in the fetus (Sheehan 2000).

Exogenous pollutants that modify endocrine system function induce documented changes in reproductive activity in wildlife species (Guillette and Gunderson 2001). Changes during fetal endocrine programming may also induce changes in growth and metabolic axes, resulting in somatic effects and metabolic disorders in adult humans (Fowden et al. 2005).

With the greater understanding of the correlation between early endocrine programming and later developmental effects, the concern over natural and synthetic estrogenic compounds in surface waters has grown over the last two decades (Jobling et al. 1995; McLachlan et al. 1984). Investigators have developed elegant analytical methods to detect low quantities of both estrogenic parent compounds and metabolites (Reddy et al. 2005), and biological screening assays to demonstrate estrogenic activity in surface waters (Witters et al. 2001). Environmental estrogens activate estrogen receptors, inducing proliferation of human breast cancer cells *in vitro* (Soto et al. 2004) and hypertrophy of mouse uterine epithelial cells *in vivo* (Markey et al. 2001), and affect gonad size and testosterone production in wild fish (Orlando et al. 2004).

As discussed above, the effects of estrogenic endocrine disruption may not be limited to reproductive endpoints. Estrogen receptors are also present in bone cells. Endogenous estrogen plays a role in regulating bone modeling by initiating expression of steroid response elements in osteoblasts and osteoclasts, and regulation of bone cell proliferation (Oursler et al. 1993). Therefore, exogenous estrogens may disrupt endogenous endocrine control of bone modeling. Migliaccio et al. (1995) reported short-term exposure to diethylstilbestrol in fetal and neonatal mice caused permanent skeletal alterations in mineralization, bone mass and bone size. Lind et al. (2004) postulated a similar change in juvenile alligator bone density to be caused by estrogenic compounds in the animals' polluted habitat.

Numerous studies report vertebral deformities in wildlife species including mammals, teleosts, and aquatic vertebrates (Kolmstetter et al. 2000; Schuytema et al. 1991). The incidence of skeletal deformities in fish species reported worldwide supports their candidacy as a biological monitor for pollution (Bengtsson 1979) and as a model for vertebral malformation studies (Fisher et al. 2003). Recent studies have demonstrated vertebral deformities resulting from 17alpha-ethinylestradiol (Boudreau et al. 2004) and 17beta-estradiol (Urushitani

et al. 2002) in the mummichog, a marine teleost. These studies suggest a possible correlation between exposure to xenobiotic estrogenic compounds and altered bone patterning and formation, adding bone to the list of target organs (reproductive, thyroid) potentially affected by endocrine disrupting chemicals.

The hypothesis for this work is that exogenous estrogenic compounds may disrupt bone tissue development and induce vertebral dysmorphogenesis in exposed juvenile fathead minnows (*Pimephales promelas*), a freshwater teleost. This study investigates the ability of two surface water xenoestrogens to induce the morphological endpoint of vertebral malformation in a bioassay at and above environmentally relevant concentrations. We used 17alpha-ethinylestradiol (EE2), a potent synthetic estrogen, to investigate the concentration-dependent morphological response, and compared effects with those elicited from similar concentrations of bisphenol A (BPA). BPA has been reported to induce developmental abnormalities in freshwater sponges (Hill et al. 2002) and *Xenopus* frog larvae (Iwamuro et al. 2003), and disrupt endocrine function in fish species (Van den Belt et al. 2003). We performed 30-day exposures beginning at the egg stage (24-48 hours post-fertilization), analyzing fish for length and microscopically for skeletal development progress and vertebral abnormalities. To determine the effects EE2 and BPA have on bone cell proliferation, we used MC3T3 murine osteoblast cells in *in vitro* assays. While teleost bone cells are not available commercially and are difficult to grow in primary culture, MC3T3 osteoblasts are suitable for estrogen-induced proliferation assay. Furthermore, these cells represent a link between mammalian bone cell *in vitro* response to hormonally active agents and the morphological and somatic responses of fish in the *in vivo* bioassays. The goal of this work is to clarify the role of exogenous estrogenic compounds in the disruption of osteoblast proliferation, and ultimately the process of vertebral dysmorphogenesis.

Materials and Methods

Chemicals

EE2 (98% purity) and BPA (99% purity) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Concentrated stock solutions were prepared with deionized water and 95% ethanol and stored at 4-6°C. Unless otherwise noted,

all reagents and supplies were purchased from VWR International (Westchester, PA, USA).

Exposures and Rearing Conditions

Fathead minnow eggs on polyvinyl chloride breeding tiles were obtained at the eyed stage from Parametrix Environmental Research Laboratory (Albany, OR, USA). One tile each was placed in 400 ml of exposure solutions (control, 0.1% ethanol solvent control-SC, and 0.1, 1, 10, 100 µg/l EE2 or 0.1, 1, 10, 100, 1000 µg/l BPA nominal concentrations) prepared with dechlorinated filtered water containing 125 µg/l Paraguard™ (formaldehyde and methanol-free, Seachem Labs, Covington, GA, USA) and concentrated stocks. Paraguard™ was added to exposure solutions (including controls) to prevent confounding fungal and bacterial contamination of eggs. Exposure solutions containing eggs were aerated with forced air through Pasteur pipets during hatch duration. 200ml fresh exposure solutions (50% renewal) were added to each egg container after 72 hours. Fish typically hatched at or around 72 hours post-exposure.

Two days post-hatch (dph), fish were transferred by plastic pipet to beakers containing fresh exposure solutions, prepared from concentrated stock solutions diluted in dechlorinated filtered water only. Three replicate beakers of n=20 fish of were prepared for each exposure concentration. Solutions were renewed daily by preparing 100 ml fresh exposure dilutions from stocks, then transferring fish by pipet, yielding approximately 100% renewal. Total exposure time for animals was 29-30 days (25+ dph). Two separate experiments for BPA and three for EE2 were performed.

Water temperature was 22-24 °C during the duration of exposure. Fish were fed spirulina algae (Algae Feast, Earthrise, Petaluma, CA, USA) daily after transfer to beakers until 5 dph. After 5 dph fish were fed live *Artemia* sp. (GSL Brine Shrimp, Ogden, UT, USA) cultured daily in the lab, plus algae as needed. Exposure solutions were aerated continuously during exposure. Water quality was checked several times weekly, with pH ranging 7.4-7.6. Nitrates were ≤0.5 mg/l, and NH_3 ≤1.0 mg/l, both rarely detected. Eggs and fish were on a 14:10 hr light: dark cycle.

Nominal concentrations of EE2 and BPA were analyzed twice during exposure duration (at or near 5 and 20 dph) using a competitive ELISA specific

for each compound (Abraxis Kits, Warminster, PA, USA). Samples from at least two different beakers of each exposure concentration were diluted (if necessary) to fall within range of analytical standards (0.05 to 3 µg/l for EE2, 0.05 to 10 µg/l for BPA).

Staining Preparation, Vertebral Analyses, and Fish Length

At 25-26 dph, fish were analyzed for vertebral abnormalities. Fish were placed in a 0.2% calcein (Sigma-Aldrich USA) solution (pH 7.4) for 10 minutes, then fresh water for 10 minutes to remove excess stain (Du et al. 2001), then in 200 mg/l MS-222 (Finquel, Redmond, WA, USA) with 200 mg/l sodium bicarbonate buffer for euthanasia. Euthanized specimens were examined by fluorescence microscopy using a Leica MZFL111 stereoscope (Bartles and Stout, Bellevue, WA, USA) equipped with a mercury lamp and fluorescein/green fluorescence protein filter. Images of each fish were recorded using Image-Pro Plus software (Media Cybernetics, Silver Springs, MD, USA). Fish were examined for vertebral malformations including lordosis, scoliotic curvatures, spinal twisting or torsion, and compressed or fused vertebrae, and recorded as deformed or not deformed (resulting in binomial data).

Fish were also examined for vertebral skeletal growth, and given a developmental score to quantitatively describe this qualitative observation. Stages of development have been similarly treated in the Carnegie stages of the human embryo, the chick embryo (Hamburger and Hamilton 1951), frog (Gosner 1960), and zebrafish (Kimmel et al. 1995). The developmental scores used here (Table 3.1) were modified from those previously described in Villeneuve et al. (2005); a median value was given to intermediate-staged fish to more accurately describe skeletal development. Healthy 25-26 dph juvenile fathead minnows possess a fully ossified vertebral column, with neural and haemal processes projecting from each vertebra (see Figure 3.1a). Disruption of full growth potential at this stage of development includes a reduction in vertebral process number and size, or more severely a reduction in the number of calcified vertebrae. After microscopic examination, fish were archived in 10% buffered neutral formalin. Lengths of fixed fish (in mm from rostral end to tail fin) were measured using a digital caliper.

Table 3.1. Developmental score classification system (from Villeneuve et al. 2005); each score characterizes vertebral growth of 25-26 day post-hatch fathead minnows.

Score	Description
1	Little to no spinal development (calcification of vertebrae to area of dorsal fin only)
2 / 2.5	Incomplete development; calcification posterior to dorsal fin / vertebra(e) immediately anterior to caudal fin not calcified
3 / 3.5	Vertebrae fully calcified / neural and haemal processes appearing
4 / 4.5	Full complement of neural and haemal process on vertebrae / processes well developed, with some branching present

Cell Proliferation Studies

MC3T3 mouse osteoblast cells were obtained from Mayo Clinic (Rochester, MN, USA). These adherent cells are easily cultured and express estrogen (among other steroid hormone) receptors (Masuyama et al. 1992). Cells were cultured at 37°C in 5% CO_2 atmosphere in α-modified minimal essential medium (BioWhittaker) containing 10% fetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin (medium).

Cells were suspended in medium and plated at a density of 1 X 10^5 cells per well in a 96-well culture dish (Falcon®). 24 hours after plating, medium was removed and 100 μl treatment solutions (medium alone or supplemented with 0.01% ethanol-SC, 10^{-11} to 10^{-5} M EE2, or 10^{-11} to 10^{-5} M BPA serial dilutions) were added. These concentrations encompass the concentrations used in the bioassays (low ng/l to low mg/l). Treatment renewal was repeated after 2 days. At five days total culture (96-hr chemical exposure), cells reached confluency and proliferation rates were measured by MTT cell proliferation assay kit (ATCC, Manassas, VA, USA).

Statistical Analysis

Significance of vertebral malformation and survival concentration-responses within treatments and difference between EE2 and BPA with respect to vertebral malformations were analyzed using logistic regression analysis due to the binomial nature of the data (SAS statistical software, Cary, NC, USA). Malformation data were subjected to logit transformation. Developmental growth in bioassays (based on the numerical score of 1-5 in Table 3.1) and fish length (normalized to percent no treatment control) were was analyzed using one-way ANOVA with Tukey-Kramer post-tests (InStat® GraphPad software, San Diego, CA, USA). MC3T3 cell proliferation rates (as represented by light absorbance) were analyzed using one-way ANOVA with InStat® software. Data in graphs is represented by means ± standard error of the mean. The threshold for statistical significance was considered to be $p < 0.05$ for all analyses.

Results

Actual concentrations of EE2 and BPA were within 0.9-1.5X nominal concentrations as determined by ELISA analyses. Nominal concentrations are therefore used in all figures and discussions. The types of malformations observed in both EE2 and BPA exposed fish include spinal curvature and torsion and vertebral bone compression and fusion (Fig 3.1b-c). A developmental score of 2.0 (few vertebrae ossified, no vertebral spines present) was also included as a vertebral malformation, as severe curvature was often associated with this score, and fish with a 2.0 score were severely retarded with regard to vertebral development compared to fish with higher scores.

EE2 Bioassay Results

EE2 caused significant increases in vertebral malformations over controls in exposed juvenile fathead minnows (Fig. 3.2a). There was no difference between results for control fish and solvent-exposed control fish. The concentration-dependent effect of EE2 was significant and non-monotonic. Greater EE2 concentrations resulted in increasing vertebral malformation

responses (0.1 to 10 µg/l) up to the highest concentration (100 µg/l), at which deformity rates decreased significantly from the previous dose.

In addition to producing vertebral column malformations, EE2 retarded vertebral development in juvenile fathead minnows. Developmental scores and lengths for EE2-exposed fish followed an inverse relationship to vertebral malformations (Fig. 3.2b). Developmental score decreased significantly from controls at 1 and 10 µg/l, but increased at 100 µg/l, the exposure concentration at which deformity rate drops. An increase in occurrence of vertebral dysmorphogenesis coincided with a decrease in the overall vertebral skeletal development in EE2 exposed fish.

Survival rates for fish exposed to EE2 were reduced from control rates, although there was no significant difference in mortality among EE2 exposure concentrations (59±3% of controls). It is unclear whether vertebral deformity played a role in the mortality of EE2-exposed fish.

BPA Bioassay Results

BPA exposure did not induce a significant concentration-dependent increase in vertebral malformations in exposed juvenile fathead minnows over control fish (Fig. 3.3a). EE2 exposure significantly increased vertebral malformation rates in developing fathead minnows over equal concentrations of BPA.

BPA did not affect developmental scores significantly in exposed fish except at the highest concentration tested, 1000 µg/l (Fig. 3.3b). The slight decrease in developmental score correlated to the relatively small increase in vertebral malformation rates as compared to control fish. This inverse relationship between developmental score and vertebral deformity followed that observed in EE2 exposures. Fish length was not significantly affected by BPA exposure.

Survival was not significantly affected by BPA exposure, and was significantly higher than survival in EE2 exposures at equal concentrations (data not shown).

Cell Proliferation Results

EE2 increased 96-hr MC3T3 osteoblast proliferation (Fig. 3.4a) at low to moderate concentrations (0.003 to 30 µg/l), although the increase was not significant.

The effect of BPA on 96-hr cell proliferation rates was similar to that of EE2, with non-significant increases at 0.0023 to 23 µg/l, and a non-significant decrease in MC3T3 cell proliferation at the highest concentration tested (2300 µg/l, 10^{-5}M).

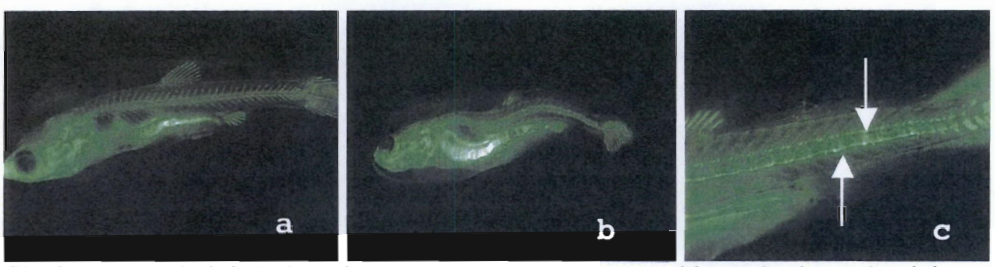

Fig 3.1. 25-26 dph fathead minnow skeletons viewed by calcein stain: (a) normal vertebral development (solvent control), developmental score 4.5; (b) vertebral curvature (10 µg/l EE2); (c) multiple bone fusions indicated by arrows (10 µg/l EE2).

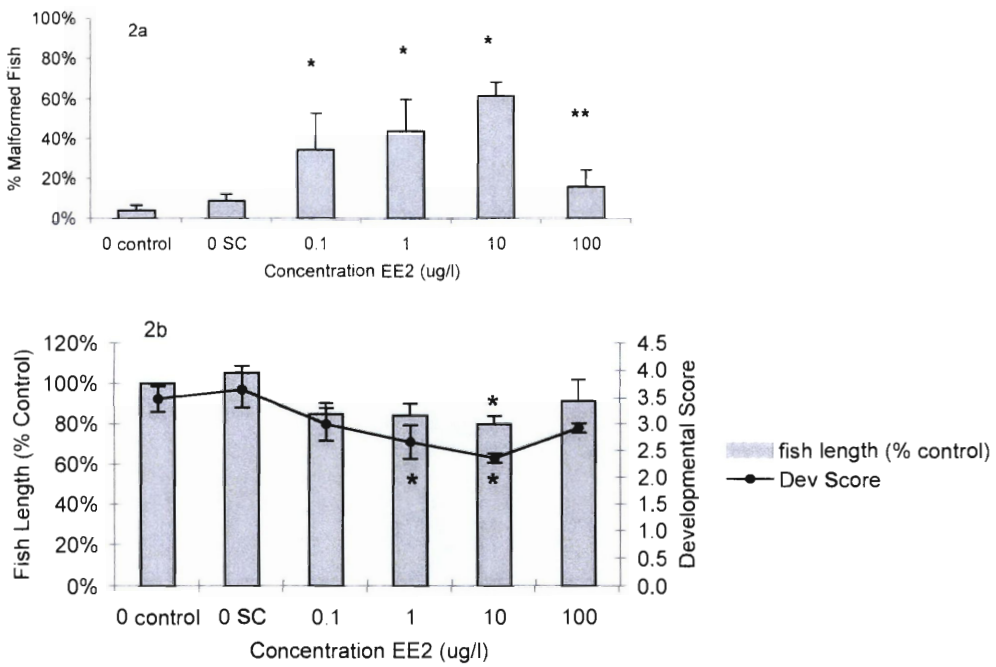

Fig 3.2. Results for juvenile fathead minnows exposed to EE2 from egg stage to 25-26 dph: (a) Vertebral malformations and (b) Fish length (% no treatment control) and developmental score. * indicates significant difference from controls ($p < 0.05$), ** indicates significant difference from 1 and 10 µg/l ($p < 0.05$)

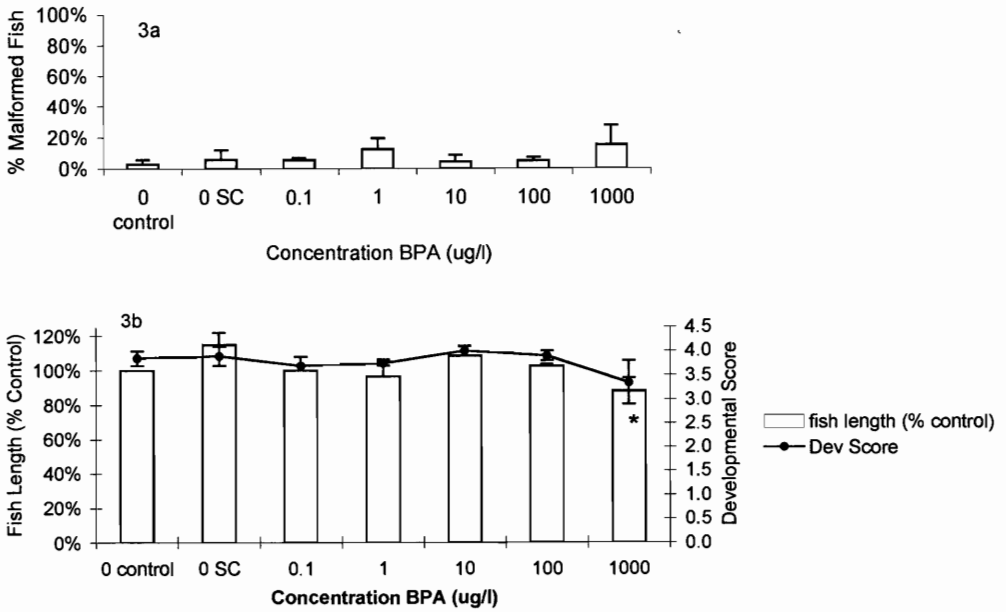

Fig 3.3. Results for juvenile fathead minnows exposed to BPA from egg stage to 25-26 dph: (a) Vertebral malformations and (b) Fish length (% no treatment control) and developmental score. * indicates significant difference from controls ($p < 0.05$)

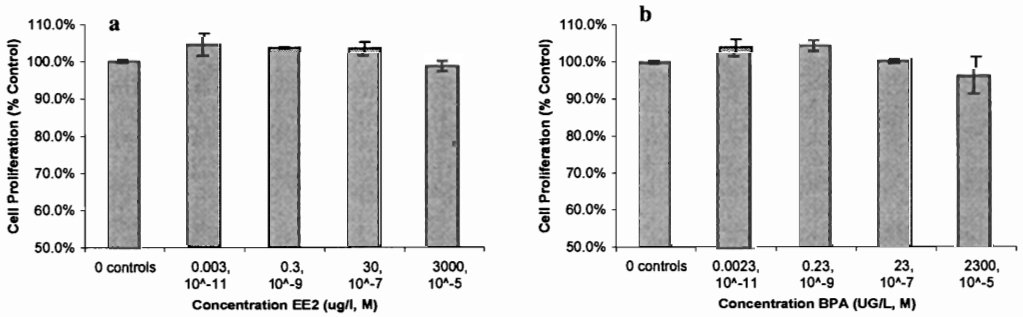

Fig 3.4. Proliferation rates for MC3T3 osteoblasts after 96-hr (a) EE2 and (b) BPA exposures

Discussion

Anthropogenic and natural compounds found in the environment have been reported to exert estrogenic activity at the cellular level, mediated by the estrogen receptor (Soto et al. 2004). Estrogen-mediated cellular proliferation effects have been demonstrated in estrogen-responsive tissues after exposure to phytoestrogens and bisphenol A (Markey et al. 2001; Pocock et al. 2002). Kanno et al. (2004) reported the proliferative effects of estrogen and estrogenic

compounds in both human breast cells and murine osteoblast cells. Estrogen receptors are present in bone tissue, and estradiol is one of the most important endogenous factors in bone growth, maintenance, and resorption. However, the potentially disruptive effects of environmental estrogens on bone formation in the whole animal have not been emphasized; the focus of estrogen-related effects in previous *in vivo* studies has been primarily on reproductive endpoints.

An aim of many endocrine disruption studies is the measurement of long-term health consequences in an organism from chemically altered endocrine function during early development (Krimsky 2001; Melnick et al. 2002). This study investigated the ability of two estrogenic compounds, EE2 and BPA, to induce the morphological endpoint of vertebral malformation in a bioassay at and above environmentally relevant concentrations. Vertebral malformations persist in affected fish throughout development; fathead minnows raised in dechlorinated, filtered, circulating water retained vertebral malformations for 9 months (approximately one-third of their life-span at time of sacrifice; data not shown). The potential long-term health and fitness consequences of this sublethal endpoint include decreases in abilities of food acquisition, predator avoidance, territory defense, and mate attraction in affected fish (reviewed by Bengtsson 1979). It is unclear whether the spinal curvature (often observed with a score of 2.0) in the juvenile fish in this study is a result of a poorly ossified and unstable vertebral column, or if poor vertebral development is a result of early spinal curvature or malformation, resulting in impaired mobility and feeding capabilities. It is also unclear whether the increased mortality in EE2-treated fish is due to these deformity-related complications as a result of endocrine disruption, or to another mechanism of estrogen toxicity.

EE2 is a primary component of contraceptive pills and is the most common estrogen found in human wastes. The ethinyl group on EE2 blocks metabolic breakdown, hindering environmental degradation (Thacker 2004). The amount of EE2 present in wastewater treatment plant effluent is typically less than 10 ng/l (Daughton and Ternes 1999; Johnson and Williams 2004; Spengler et al. 2001). However, effluent from concentrated animal feeding operations or waste storage/treatment centers for dairy and swine may reach ten times the levels of estrogens in human waste (Raman et al. 2004). We induced vertebral malformation in juvenile fish significantly over controls at estrogen concentrations

that are at and above those reported in the environment. In a similar study, spinal deformities in the mummichog coincided developmentally with ossification of the vertebral bone (Boudrea et al. 2004). Researchers have reported reproductive endocrine disruption in multiple fish species at EE2 concentrations similar to those used in these bioassays (MacLatchy et al. 2003; Schultz et al. 2003) and lower concentrations (Fenske et al. 2005). The non-monotonic concentration response for EE2-induced vertebral dysmorphogenesis reported here is similar to that for several endocrine disruption endpoints (Melnick et al. 2002), and may be correlated to the decreased bone-specific efficacy and increased toxicity of estrogen (and increased mortality) at higher concentrations (Welshons et al. 2003), although the role of vertebral dysmorphogenesis in fish mortality in this study is not clear.

The concentrations of BPA used in these bioassays represent those found in surface waters (ng/l to µg/l) and higher (Jin et al. 2004). BPA did not significantly induce vertebral malformations or impair skeletal development or survival at environmentally relevant concentrations. BPA is a component of resin-based and plastic products, and BPA exposure in aquatic vertebrates and in humans may come from numerous sources (Bae et al. 2002; Rodriguez-Mozaz et al. 2004). In a recent study of 394 US adults, the 95th percentile urine concentration was 5 µg/l (Calafat et al. 2005). As discussed, BPA has a significantly lower affinity for the estrogen receptor than EE2 or endogenous estrogen ligands, and several putative environmental estrogens have a similar low relative binding efficiency for the fathead minnow estrogen receptor (Denny et al. 2005). Despite its reduced potency in these bioassays, the potential risk BPA (or similar "weak" estrogens) may pose to wildlife and human health through additive effects with more potent estrogens (Pocock et al. 2002) is unknown. Brian et al. (2005) demonstrated that several estrogenic toxicants (including BPA) act together in a predictable additive manner to produce effects *in vivo* when they are present in multichemical mixtures at concentrations that are not individually efficacious.

The reduced ability of BPA to induce vertebral malformation with respect to EE2 may be a result of possible differences in its uptake, distribution, and metabolism, or in part to its 10^3-10^4 times lower affinity for the estrogen receptor (Matthews et al. 2000; Rich et al. 2002). Here, the only significant increase in

vertebral deformity associated with BPA exposure occurs at a concentration that is 10^4 times higher than the lowest concentration of EE2 tested. The difference between the abilities of EE2 and BPA to induce vertebral dysmorphogenesis may also result from the modulating capacity of plasma steroid binding plasma proteins (SBPs). SBPs protect endogenous steroids from rapid metabolic degradation, and may be involved in signal transduction to nuclear steroid receptors. The affinity of EE2 for fish SBPs is over 400 times greater than BPA (Tollefsen 2002). Milligan et al. (1998) suggest environmental estrogens that poorly bind SBPs are unlikely to produce biological effects in humans and fish unless present in very high concentrations. Although unbound estrogenic ligands in plasma more readily bind estrogen receptors (Csanády et al. 2002), unbound BPA is rapidly metabolized and excreted in rats and humans (Kurebayashi et al. 2005, Teeguarden et al. 2005) and in fish (Lindholst et al. 2001, 2003).

The prevalence, economic cost, and quality of life issues associated with vertebral malformations in humans and their occurrence in wildlife species suggests the necessity of an animal model to study the etiology, development, and risks of these types of disorders. The first National Health and Nutrition Examination Survey published in 1987 documented a scoliosis prevalence rate of 8.3% among US adults (Carter and Haynes). Today, idiopathic scoliosis is a condition that affects two to three percent of people worldwide (an estimated 6 million Americans), emerging by adolescence. Many vertebral deformities in wildlife (including teleosts) and humans are sublethal conditions of generally idiopathic etiology, and this work serves to demonstrate bone growth perturbation by estrogenic compounds present in surface waters. The fathead minnow was used in the bioassay due to its relatively fast vertebral development and background as a model in toxicity studies. The teleost model has been used to study another human bone dysmorphogenesis disorder, osteogenesis imperfecta (Fisher et al. 2003). Furthermore, teleosts are a sensitive organism found *in* the environment where they are directly exposed to toxic substances that may also negatively affect human health.

The outcomes of this study suggest spinal malformations in vertebrates may result from exposure to potent environmental estrogenic agents during early development. "Weak" estrogenic compounds such as BPA – those with an affinity for the estrogen receptor orders of magnitude below estradiol – do not pose a

similar risk, although weak estrogens in additive mixtures within surface waters have the potential to contribute to the development of vertebral dysmorphogenesis. The non-significant changes in osteoblast *in vitro* response to estrogenic compound exposure did not support cell proliferation as the sole mechanism of bone dysmorphogenesis observed in the bioassays. In a review of *in vitro* estrogenic assays, Scrimshaw and Lester (2004) discuss the limitations of proliferation assays as definitive indicators of biological endocrine disruption; the authors emphasize the need for multi-level screening assays. Markey et al. (2001) reported subtle changes in mouse uterine epithelial cell morphology at 5, 75, and 100 mg/kg BPA, but observed a uterotrophic response (an increase in uterine wet weight) at 100 mg/kg only. Bone modeling cells are controlled by multiple factors, and *in vitro* proliferative response to estrogenic agents may be influenced differently from the milieu of endocrine signals *in vivo*. Osteoblast cell responses to endogenous estrogen include induction of steroid response element genes and subsequent protein expression. Further studies to elucidate perturbation of homeostatic endocrine signaling during bone growth should include growth factor signaling disruption and receptor expression in osteoblasts and fathead minnows exposed to the xenoestrogens EE2 and BPA.

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INSULIN-LIKE GROWTH FACTOR-1 AND IMPAIRED DEVELOPMENT IN JUVENILE FATHEAD MINNOWS (*PIMEPHALES PROMELAS*) EXPOSED TO 17ALPHA-ETHINYLESTRADIOL

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Chapter 4: Insulin-Like Growth Factor-1 and Impaired Development in Juvenile Fathead Minnows (*Pimephales promelas)* Exposed to 17Alpha-Ethinylestradiol

Abstract

Natural and synthetic estrogens detected in surface waters have been linked to endocrine signaling disruption in several species. Endogenous estrogen is a proliferation-inducing compound in osteoblasts, influencing cartilage and bone deposition during development in vertebrates. The aim of this study was to investigate the effects of exogenous estrogen exposure on expression of an essential endocrine signal in bone formation, insulin-like growth factor-1 (IGF-1). We hypothesize that xenobiotics with estrogenic activity adversely impact vertebral bone formation through disruption of IGF-1 signaling. We exposed fathead minnows (*Pimephales promelas*) to 0.1-100 µg/l 17α-ethinylestradiol (EE2) and 0.1-1000 µg/l bisphenol A (BPA) from egg stage to 25-26 days post-hatch. Fish were weighed and processed to quantify IGF-1 levels in whole body homogenate. In addition to the *in vivo* assays, MC3T3 osteoblasts were used to characterize *in vitro* bone cell IGF-1, its receptor (IGF-1R), and estrogen receptor (ER) expression in response to EE2 and BPA exposure. EE2 and BPA treatment reduced IGF-1and IGF-1R protein levels *in vitro*, although these trends were not statistically significant. A concentration-dependent decrease in fish IGF-1 concentration significantly correlated to decreased weights in EE2-exposed fish, but when normalized for total protein IGF-1 levels were not significantly reduced from controls. BPA did not cause significant changes in weights of exposed fish compared to control fish. Although EE2 concentration-dependent decreases in skeletal development have been observed in fathead minnows, the data reported here does not support IGF-1 disruption as the mechanistic basis of vertebral dysmorphogenesis.

Introduction

Environmental estrogens have the potential to induce changes in homeostatic endocrine signaling in a variety of tissues and animals. While the focus of estrogen-related endocrine disruption has primarily been on reproductive

endpoints, endogenous estrogen signaling is also essential in bone development. Disruption in homeostatic bone formation during fetal or juvenile development may result in altered bone density, size, and strength (Ammann et al. 1996, Stokes et al. 1996). Perturbations in endocrine programming during early development of an organism may result in somatic effects still evident in an adult (Fowden et al. 2005).

Laboratory exposures of aquatic vertebrates to estrogenic compounds have resulted in vertebral anomalies (Warner and Jenkins (unpublished results), Urushitani et al. 2002), and several studies link estrogenic compound exposure in a developing organism to bone morphogenesis disruption (Lind et al. 2004; Migliaccio et al. 1995; Sonne et al. 2004). Boudreau et al. (2004) report the development of vertebral deformities coinciding temporally with ossification. However, the mechanism involved in bone dysmorphogenesis from exogenous estrogens is not clear.

Multiple endocrine signals, along with the molecular and cellular events involved in osteogenesis and bone remodeling have been characterized in vertebrates (see Cancedda et al. 2000 and van der Eerden et al. 2003 for reviews). Of particular interest is insulin-like growth factor-1 (IGF-1), a 7-7.5kDA somatomedin peptide that has been demonstrated to be a critical endocrine signal for bone development. IGF-1 is produced in the liver in a paracrine response to growth hormone (GH), and circulates in the bloodstream bound by insulin-like growth factor binding proteins (IGFBPs). The IGF-1 receptor (IGF-1R), upon IGF-1 binding, induces mitogenic activity in numerous tissues through tyrosine kinase-regulated transcription factors (Adolphe 1992). Bone-forming chondrocytes and osteoblasts produce IGF-1 in an autocrine response to IGF-1 from the blood. IGF-1 stimulates collagen synthesis and inhibits collagen degradation (Zofkova 2003), and improves the conditions for bone mineralization (Sathanogopal and Dixon 1999).

IGF-1 structure, function, and regulation are highly conserved in mammals and fish, and IGF-1 protein activity has been reported in a number of fish species. While IGF-1 and its receptor are present in several tissues, IGF-1 is most highly expressed in liver (reviewed by Duan 1998). IGF-1 mRNA and IGF-1R protein expression have been characterized in zebrafish embryos using *in situ* hybridization and immunohistochemistry, respectively (Maures et al, 2002). Blood

or tissue levels of IGF-1 and its mRNA positively correlate with nutrition and growth rate in teleosts (reviewed by Reinecke et al. 2005). Juvenile coho salmon treated with IGF-1 for several weeks significantly increase linear and weight growth (McCormick et al. 1992). IGF-1 produced locally in osteoblasts of the axial skeleton in mice is essential for vertebral growth (Lupu et al. 2001). Likewise, local IGF-1 signaling is critical in Atlantic salmon vertebral development; up-regulation of IGF-1R gene expression precedes increased vertebral bone density (Wargelius et al. 2005).

Estrogen is an endogenous endocrine signal that affects IGF-1 signaling. Studies in mammals demonstrate the "early and predominant" effects of estrogen on bone modeling: a decrease in the amount of bone resorption and proliferation and synthesis of enzymes and bone matrix proteins in osteoblast-like cells (Oursler et al. 1993). Estradiol increases IGF-1 gene expression in rodent osteoblasts, and treatment of a human fetal osteoblast cell line with high estrogen receptor levels increases steady state levels of IGF-1 mRNA in a time- and dose-dependent fashion, suggesting the IGF-1 gene is a target for estrogen action (an estrogen response element) and that IGF-1 may mediate estrogen effects in bone (Kassem et al, 1997).

We hypothesize exogenous estrogenic compounds affect IGF-1 protein expression and activity at bone tissue, and impair growth in an exposed vertebrate model through the disruption of homeostatic IGF-1 signaling. We exposed fathead minnows (egg stage to 25 days post-hatch) to two putative estrogenic endocrine disruptors documented in surface waters, EE2 and BPA, and analyzed whole body concentrations of IGF-1 through radioimmunoassay (RIA). We also performed immunocytochemistry (ICC) on murine osteoblasts to determine IGF-1, IGF-1R, and estrogen receptor (ER) protein expression after EE2 and BPA exposure. Teleost osteoblasts are not commercially available and are difficult to grow in primary culture; however the mouse osteoblast cell line (MC3T3) may suitably link the effects of putative estrogenic compounds *in vitro* in mammalian bone cells to *in vivo* effects in teleosts.

This work proposes a mechanism of bone signaling perturbation by potent exogenous estrogens, and links a biochemical endpoint to the morphological endpoints of impaired development described in this study and vertebral deformity elicited in a previously reported companion bioassay.

Materials and Methods

Chemicals

EE2 (98% purity) and BPA (99% purity) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Concentrated stock solutions were prepared with deionized water and 95% ethanol and stored at 4-6 °C. Unless otherwise noted, all reagents and supplies were purchased from VWR (Westchester, PA, USA).

Fish Exposure and Rearing Conditions

Fathead minnow eggs on polyvinyl chloride breeding tiles were obtained at the eyed stage from Parametrix Environmental Research Laboratory (Albany, OR, USA). One tile each was placed in 400 ml of exposure solutions (no treatment control, 0.1% ethanol solvent control, and 0.1, 1, 10, and 100 µg/l EE2 or 0.1, 1, 10, 100, or 1000 µg/l BPA nominal concentrations) prepared with concentrated stocks diluted in dechlorinated filtered water containing 125 µg/l Paraguard™ (formaldehyde and methanol-free, Seachem Labs, Covington, GA, USA). Paraguard™ was added to exposure solutions (including controls) to prevent confounding fungal and bacterial contamination of eggs. Exposure solutions containing eggs were aerated with forced air through Pasteur pipets during hatch duration. Fresh 200 ml exposure solutions (50% renewal) were added to each egg container after 72 hours. Fish typically hatched at or around 72 hours post-exposure.

Two days post-hatch (dph), fish were transferred by plastic pipet to beakers containing fresh exposure solutions, prepared from concentrated stock solutions diluted in dechlorinated filtered water only. A minimum of three replicate beakers of n=20 fish or n=30 fish were prepared for each exposure concentration for each experiment (one BPA and two EE2 exposures). Solutions were renewed daily by preparing 100 ml fresh exposure dilutions from stocks and dechlorinated filtered water, then transferring fish by pipet, yielding approximately 100% renewal. Total exposure time for animals was 29-30 days (25+ dph).

Water temperature was 22-24 °C during the duration of exposure. Fish were fed spirulina algae (Algae Feast, Earthrise, Petaluma, CA, USA) daily after transfer to beakers until 5 dph. After 5 dph fish were fed live *Artemia* sp. in excess (GSL Brine Shrimp, Ogden, UT, USA) cultured daily in the lab, plus algae

1-2 times weekly. Exposure solutions were aerated continuously during exposure. Water quality was checked several times weekly, with pH ranging 7.4-7.6. Nitrates were ≤0.5 mg/l, and NH_3 ≤1.0 mg/l, both rarely detected. Eggs and fish were on a 14:10 hr light: dark cycle. Feeding was suspended 24 hr prior to sampling.

Nominal concentrations of EE2 and BPA were analyzed twice during exposure duration (at or near 5 and 20 dph) for all experiments using a competitive ELISA specific for each compound (Abraxis Kits, Warminster, PA, USA). Samples from at least two different beakers of each exposure concentration were diluted (if necessary) to fall within range of analytical standards (0.05 to 3 µg/l for EE2, 0.05 to 10 µg/l for BPA); all samples were prepared as 10% ethanol. Actual concentrations were within 0.9-1.5X nominal concentrations.

After 25+ dph, fish from each replicate were counted and collectively euthanized in 200 mg/l MS-222 (Finquel, Redmond, WA, USA) with 200 mg/l sodium bicarbonate buffer. Pooled fish were fast-frozen in methanol over dry ice and stored at -80 °C for RIA assays.

Fish Weight and IGF-1 Analysis by Radioimmunoassay

IGF-1 protein levels in juvenile fish in response to estrogenic exposure were measured using radioimmunoassay (RIA). Serum analysis is not possible in 25 dph fathead minnows due to fish size, and IGF-1 concentrations from tissues other than serum were of interest in this study. Therefore, methods for whole body homogenate preparation were adapted from Mylchrest et al (2003) and Andersen et al (2003). All fish were weighed prior to analysis; several replicates of exposed fish were used for RIA method optimization. Frozen juvenile minnows (pooled from each exposure replicate) were thawed and weighed in a tared microcentrifuge tube, then homogenized in cold (4 °C) phosphate buffered saline with 0.1% Tween 80 at a volume up to twenty times their collective weight. The homogenate for each replicate was centrifuged at 16,000 rcf, 4 °C for one hour.

Acid-ethanol extraction of homogenate supernatant was used to separate IGF-1 from associated IGFBPs. Samples and standards of known fish IGF-1 concentration were analyzed using [125]-I radiolabeled fish IGF-1 tracer in an RIA

kit (GroPep, Adelaide, Australia), with appropriate quality controls used, including replicates and adult fathead minnow serum samples. The concentration of IGF-1 in the homogenate supernatant samples was determined by interpolation from the standard curve.

Total protein for pooled fish from each exposure replicate was analyzed using a bicinchoninic acid (BCA) biuret reaction; the colorimetric reaction is directly related to the amount of protein in a sample. BCA was added to diluted homogenate supernatant samples and known concentrations of albumin in a 96-well plate (Falcon®), incubated at 37 °C for 30 min, and read on a SpectraMax 190 microplate reader with SoftMax Pro software (Molecular Devices Corp., Sunnyvale, CA, USA). The concentration of total protein in the homogenate supernatant samples was determined by interpolation from the albumin standard curve.

In Vitro Exposures and Immunocytochemical Analysis

MC3T3 mouse osteoblast cells were obtained from Mayo Clinic (Rochester, MN, USA). These adherent cells are easily cultured and express estrogen (among other steroid hormone) receptors (Masuyama et al. 1992). Cells were cultured at 37 °C in 5% CO_2 atmosphere in α-modified minimal essential medium (BioWhittaker) containing 10% fetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin (media).

Cells were suspended in media and plated at a density of 1.75 X 10^4 cells per chamber of a 16-well Nunc Lab-Tek chamber slide (Fisher Scientific, Houston, TX, USA), and allowed to adhere overnight. The following day media was removed and 200 µl treatment solutions (media alone or supplemented with 0.01% ethanol, EE2, or BPA serial dilutions at or below bioassay concentrations) were added. Treatment renewal was repeated every two days for a total of 10 days, to simulate the chronic exposure performed in the fish bioassay and to allow the cells to reach confluence. Three separate replicates of each treatment were prepared for ICC assay. Media was removed and cells were fixed with 10% neutral buffered formalin for 20 min.

Fixed cells were rinsed with phosphate buffered saline (PBS) and blocked for up to 1 hr with 5% normal serum (specific to the secondary antibody) in PBST

(PBS with 0.2%Triton X-100 and 1% albumin). Cells were incubated overnight at 4 °C with primary antibodies diluted in PBST: 1 µg/100µl rabbit anti-IGF-1 (validated for human, rat, and fish IGF-1, GroPep, Adelaide, Australia), 2 µg/ml rabbit anti-ER (validated for human and mouse ERα, Upstate Cell Signaling Solutions, Charlottesville, VA, USA), or 1 µg/100µl chicken anti-IGF-1R (validated for human and mouse IGF-1R α-subunit, Upstate). Cells were washed with PBS and incubated at room temperature for 2 hr with appropriate secondary antibodies diluted in PBST: 5 µg/ml donkey anti-rabbit IgG conjugated with Alexa 488 or 5 µg/ml goat anti-chicken IgG conjugated with Alexa 555 (both from Molecular Probes/Invitrogen, Eugene, OR, USA). After secondary antibody incubation, cells were washed in PBS, and labeled with 1µg/ml Hoechst nuclear stain (Molecular Probes) for 10 minutes, then washed with PBS.

Chamber attachments were removed, and cells were mounted in Fluoromount G and covered with a glass cover slip. Slides were immediately examined with a Leica DMRB compound microscope (Bartles and Stout, Bellevue, WA, USA) equipped with a mercury lamp and fluorescence filters. Images and fluorescence data were obtained using Image-Pro Plus software (Media Cybernetics, Silver Springs, MD, USA). Pixel intensity (fluorescence units) per image was divided by the count of cells per image to yield a semi-quantitative analysis of pixel intensity per cell per treatment replicate (excitation and emission parameters were obtained for (no treatment) control cells and held constant for all treatment wells on each slide). Values were averaged from three separate replicates and normalized to percent (no treatment) control. Co-localization of ER, IGF-1, or IGF-1R with the Hoechst nuclear label, or IGF-1 with IGF-1R, was derived from the Pearson correlation coefficient calculated from each pair of fluorescent labels. Co-localization within specific cellular compartments (nuclear and cytosolic) was verified with a Zeiss LSM 510 Meta confocal microscope and LSM software (Zeiss, Thornwood, NY, USA).

Statistical Analyses

All statistical analyses were performed using InStat® or Prism GraphPad software, San Diego, CA, USA. For the bioassays, EE2- and BPA-exposed fish weight and IGF-1 content were normalized to percent (no treatment) control.

Statistical difference between (no treatment) control and solvent control was analyzed by t-test; when no significant difference existed between the two control groups their values were pooled within each experiment. Treatment and concentration responses were determined using one-way ANOVA with Tukey-Kramer/t-test post-tests. Linear regression was used to determine correlation between weight and ng IGF-1 per fish normalized to percent (no treatment) control.

For the ICC study, pixel intensity per cell was normalized to percent (no treatment) control for each fluorescently labeled protein at each EE2 or BPA exposure concentration and for solvent control. Statistical difference between (no treatment) control and solvent control for fluorescence of each antibody-labeled protein was analyzed by t-test; when no significant difference existed between the two control groups their values were pooled. Semi-quantitative analyses of protein fluorescence and co-localization coefficients for proteins for each treatment were analyzed using one-way ANOVA with Tukey-Kramer post-test.

Data in graphs is represented by means ± standard error of the mean. The threshold for statistical significance was considered to be $p \leq 0.05$ from controls for all analyses.

Results

Fish Weight

There was a significant increase in weight of solvent control fish over (no treatment) controls; statistical analyses to assess concentration-dependent weight response compared treatment groups to solvent control fish. Juvenile fathead minnows weighed significantly less after 30 days of EE2 exposure as compared to control fish; however, BPA treatments did not significantly affect fish weight in the 30 d exposure period (see Figure 4.1).

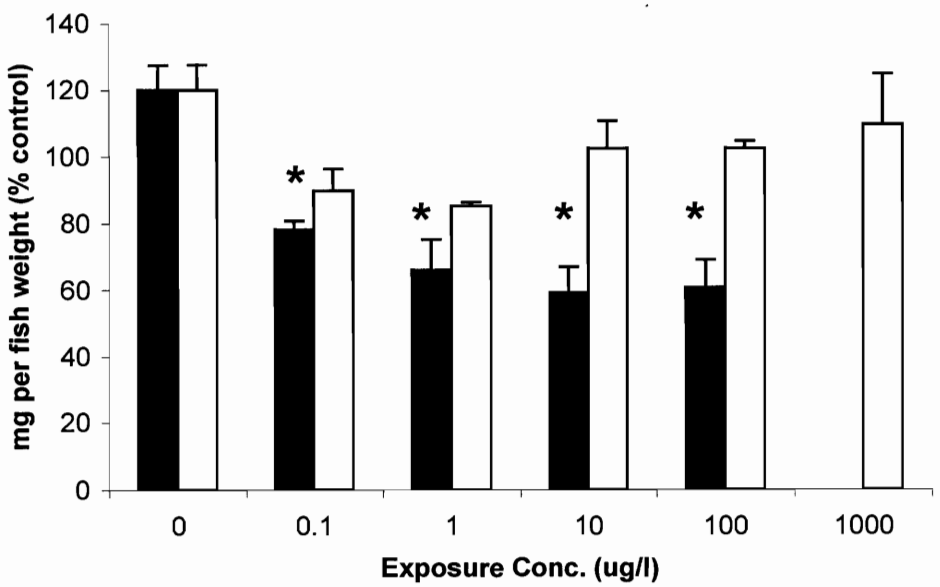

Fig 4.1. Weight of fish after two 30 d exposures of 0 (solvent control) to 100 µg/l EE2 (black bars), and one 30 d exposure of 0 (solvent control) to 1000 µg/l BPA (white bars). Fish weights are calculated from pooled weight divided by number of fish per replicate, normalized to % (no treatment) control. * Indicates significant difference from solvent control ($p < 0.05$).

Fish IGF-1 Concentration

Fish were pooled from each exposure replicate for each chemical exposure. Both IGF-1 per total protein (pooled fish) and IGF-1 per fish were calculated for each replicate and normalized to percent (no treatment) control. There were no significant differences between (no treatment) control and solvent control data; these values were combined for ANOVA analyses. EE2-exposed fish experienced a decrease in ng IGF-1/µg total protein, although this decrease was not significant (Figure 4.2). EE2-exposed fish also demonstrated a significant concentration-dependent decrease in ng IGF-1/fish as compared to control fish (Figure 4.3). Fish weight (mg per fish, normalized to percent control) was positively and significantly correlated ($r^2 = 0.971$) to IGF-1 concentration per fish (ng IGF-1/fish, normalized to percent control). BPA treatment did not significantly affect fish IGF-1 concentration (ng IGF-1/ µg total protein or ng IGF-1/fish, normalized to percent control) in the 30 d exposure period (data not shown).

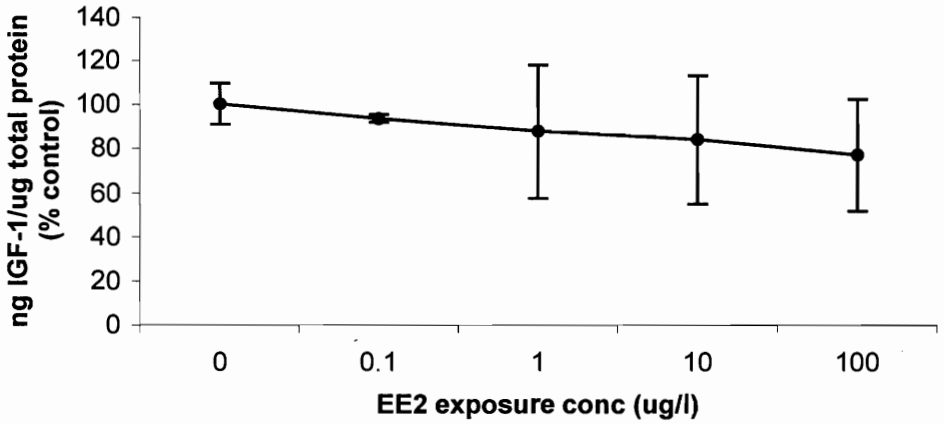

Fig 4.2. IGF-1 normalized to total protein (ng IGF-1/µg total protein) per replicate of fish after 30 d EE2 exposures. Zero concentration includes no treatment control and solvent control groups.

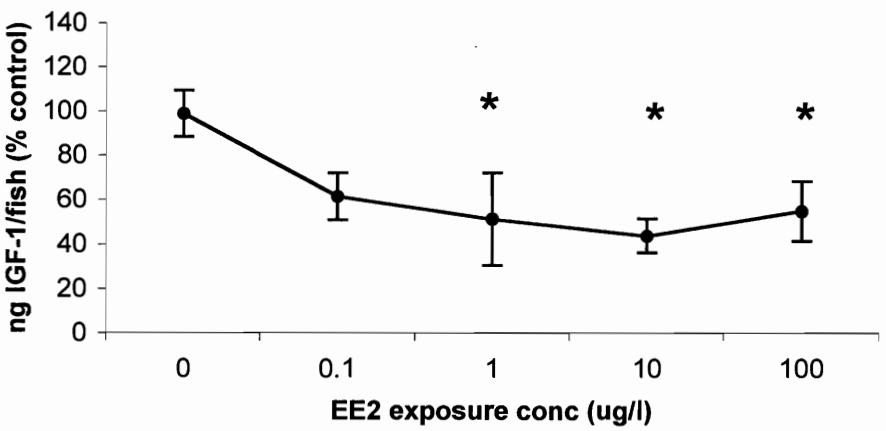

Fig 4.3. IGF-1 concentration per fish (ng IGF-1/fish) after 30 d EE2 exposures. Zero concentration includes (no treatment) control and solvent control groups. IGF-1 per fish is calculated from pooled ng IGF-1 divided by number of fish per replicate, normalized to % (no treatment) control. * Indicates $p < 0.05$ from controls.

Effects of EE2 and BPA exposure on IGF-1, IGF-1R, and ER distribution in MC3T3 osteoblast cells

Images of labeled MC3T3 mouse osteoblasts are shown in Figure 4.4. Based on the lack of labeling in negative controls (Fig. 4.4 panels a and e, pixel intensity per cell is ≤0.3% of no treatment control for each immunoreactive protein), the primary antibodies against ER alpha, IGF-1, and IGF-appeared to have appropriately labeled their antigens.

Semi-quantitative analysis of fluorescence for each treatment suggests an increase in ER (Figure 4.5a and Figure 4.6) and decrease in IGF-1R (Figure 4.5b) in response to EE2 treatment. Immunoreactive IGF-1 also increased in response to 10^{-11} M and 10^{-9} M EE2, but decreased at 10^{-7} M (Figure 4.5c). However, these trends are not statistically significant by ANOVA analysis. Trends for changes in immunoreactive protein intensities for BPA treatments are less clear due to high variability of pixel intensity per cell values.

Immunoreactive IGF-1R did not co-localize with the Hoechst nuclear label (mean Pearson coefficient of correlation = 0.48±0.03 for all treatments), and therefore represents the cytosolic cellular compartment. Neither EE2 nor BPA had a statistically significant effect on the distribution of immunoreactive IGF-1 in the nuclear or cytosolic compartments according to semi-quantitative analyses (data not shown).

Fig. 4.4. Fluorescently labeled antibodies against IGF-1, IGF-1R, and ER alpha in fixed MC3T3 osteoblasts after 10 d EE2 or BPA exposure. Panels (a) and (e) represent negative controls for ER alpha and IGF-1/IGF-1R, respectively. Immunoreactive ER alpha (green), or IGF-1 (green)/IGF-1R (red) is depicted in panels following treatment with solvent control (b, f), 10^{-7} M EE2 (c, g), and 10^{-7} M BPA treatment (d, h). Yellow color represents cytosolic co-localization of IGF-1 and its receptor following merger of IGF-1 and IGF-1R images. All images were taken at 40X magnification; scale bars represent 50 μm.

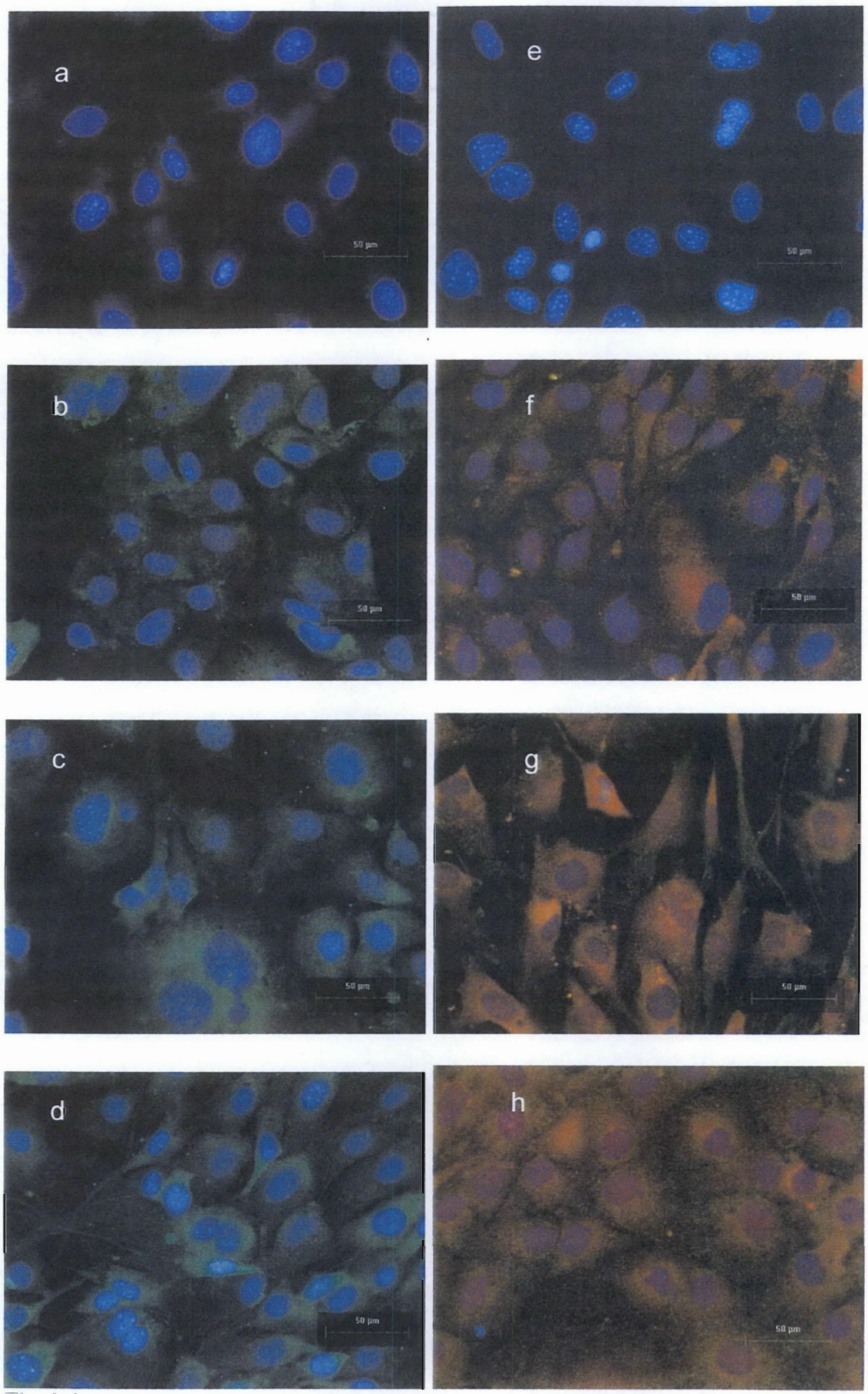

Fig 4.4

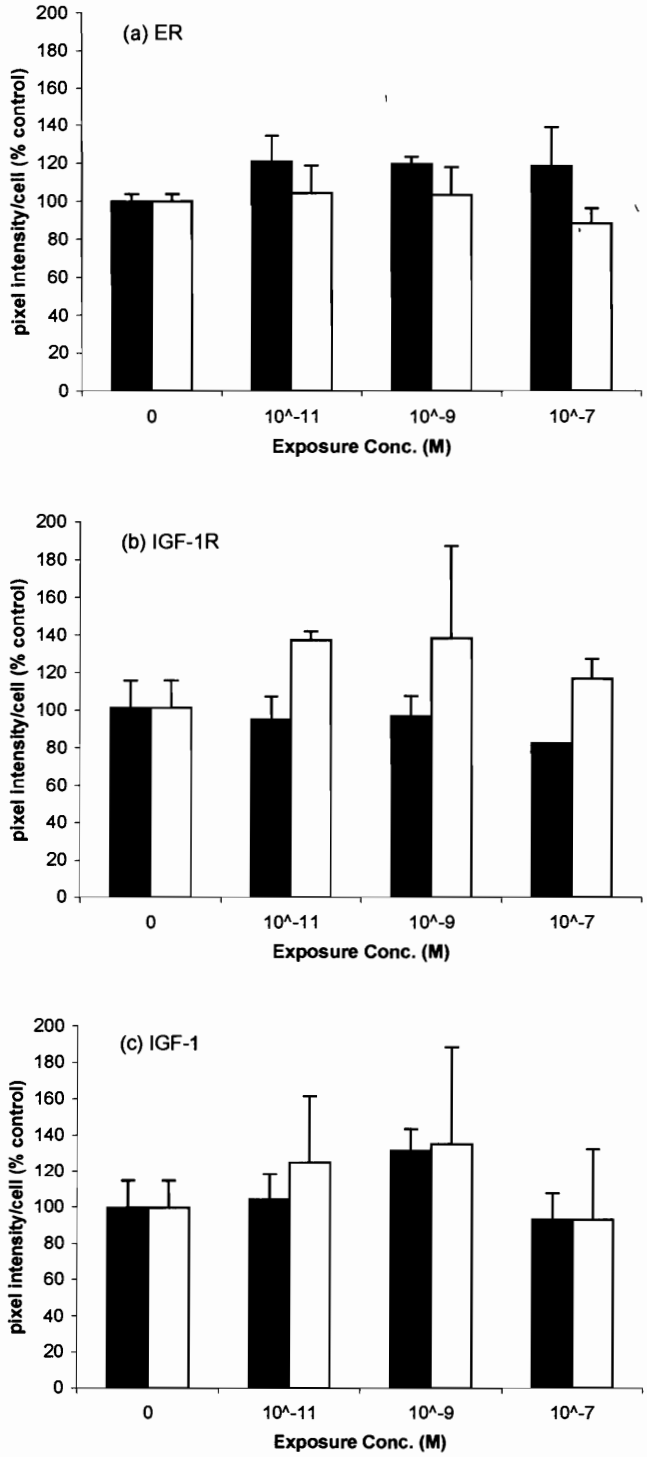

Fig 4.5. Immunoreactive (a) ER alpha, (b) IGF-1R, and (c) IGF-1 in fixed MC3T3 osteoblast cells after 10 d EE2 (black bars) or BPA (white bars) exposure. Zero exposure concentration includes (no treatment) control and solvent control. Pixel intensity per image was divided by the number of cells per image to yield a semi-quantitative analysis of pixel intensity per cell per treatment replicate.

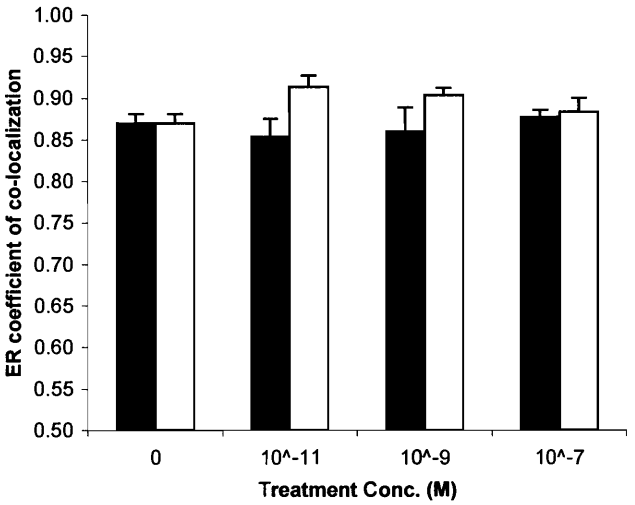

Fig 4.6. Co-localization between immunoreactive ER and Hoechst nuclear label in fixed MC3T3 osteoblast cells after 10 d EE2 (black bars) or BPA treatment (white bars). Zero exposure concentration includes (no treatment) control and solvent control. Co-localization was derived from the Pearson correlation coefficient calculated from each pair of fluorescent labels; a coefficient of 1.0 represents perfect positive correlation.

Discussion

The aim of this study was to investigate the potential perturbation of IGF-1 growth factor signaling from estrogenic compounds *in vivo* in a teleost vertebrate model. The relationship between endogenous estrogen and IGF-1 in bone tissue is one that promotes growth through chondrocyte and osteoblast stimulation. The anabolic and maintenance functions of these endocrine signals may be modified, however, by disruption of homeostatic biochemical signal regulation. The role of decreased estrogen signaling has been well documented in human osteoporosis, and impaired IGF-1 signaling in humans results in diminished bone growth capacity. IGF-1 has been used pharmacologically to promote statural growth in GH-insensitive patients (Underwood et al. 1999), and IGF-1 as a part of hormone replacement therapy is particularly beneficial in treatment of bone loss disorders in both men and women (Agnusdei and Gentilella 2005, Legroux-Gerot et al. 2005).

The role of IGF-1 in non-human vertebrates has also been studied, both to clarify IGF-1 activity and to elucidate potential alterations in bone morphogenic

processes. IGF-1 treatment increases bone mineral content and bone growth and strength, and prevents bone loss in ovariectomized rats (reviewed by Kasukawa et al. 2004). Erdmann et al. (1998) reported slightly supraphysiological estradiol increased the concentration of IGF-1 in bone matrix by up to 44.2% in ovariectomized rats. Similarly, Kanno et al. (2004) and Masuyama et al. (1992) reported increases in MC3T3 mouse osteoblast proliferation rates in response to low (sub- and physiological) 17β-estradiol (E_2) concentrations (10^{-11} to 10^{-8}M) *in vitro*. However, the proliferation-stimulating ability of E_2 decreased partially or completely in both studies at increased concentrations (10^{-7}M, or approximately 30 µg/l). E_2 has no effect on proteoglycan synthesis in bovine or teleost cartilage at physiologically relevant concentrations, but is inhibitory at higher concentrations (Mackintosh and Mason 1988, Ng et al. 2001). High-dose estrogen has been shown to down-regulate its own receptor *in vivo* in mammary tissue of lactating rats (Hatsumi and Yamamuro 2006), and in cultured human osteoblasts, resulting in reduced collagen synthesis (Ireland et al. 2002).

The *in vitro* assays in this study demonstrate ER in both the nuclear and cytosolic compartments of MC3T3 osteoblasts, similar to previous reports (reviewed by Levin 2005). Here, estrogen receptors of MC3T3 mouse osteoblasts did not down-regulate after 10 d estrogenic compound exposure. IGF-1 was localized in both the cytoplasm and nucleus as well, and may have been co-transported to the nucleus with an IGFBP (Li et al. 1997). Immunoreactive IGF-1 pixel intensity per cell increased at EE2 concentrations similar to physiological estradiol levels in fish (Wu et al. 2003), but decreased at the supraphysiological (10^{-7} M, or 30 µg/l) concentration. IGF-1R similarly decreased at supraphysiological EE2 concentration; however, these trends were not statistically significant. The distribution of immunoreactive ER, IGF-1, and IGF-1R in nuclear and cytosolic cellular compartments did not change with physiological or supraphysiological concentrations of estrogens.

The decreased concentrations of IGF-1 in fish exposed chronically to EE2 significantly correlate to decreases in body weight. The reduced IGF-1 levels and decreased weight observed in this bioassay also correlate to EE2 concentration-dependent decreases in skeletal development (r^2=0.937 and 0.840, respectively, all values normalized to percent control) in juvenile fathead minnows in a companion study (see Chapter 3). However, IGF-1 was not significantly reduced

from controls when normalized to total protein content of fish whole body homogenate, and it is unclear whether reduced fish weight is a result of IGF-1 disruption from EE2, or if IGF-1 reduction is based on overall decreased mass of fish. Therefore the data presented here do not support IGF-1 disruption as the mechanistic basis of impaired skeletal development in EE2-exposed juvenile fathead minnows.

Limitations of this study include the inability to measure IGF-1 levels in bone or liver tissue specifically, rather than whole body homogenate, due to constraints from fish size at 25 days post-hatch. EE2 did not induce *in vitro* down-regulation of ER alpha in this study, and the effect on ER beta, present in mammalian bone cells (Onoe et al. 1997), is unknown. The functional role of ER beta in the human growth plate is also unclear (Sävendahl 2005). Furthermore, teleosts express a third distinct estrogen receptor (Hawkins and Thomas 2004), although the distribution and activity of these receptors in fish bone tissue are currently unknown. The autocrine IGF-1 response to prolonged estrogen stimulation in osteoblasts may cause down-regulation of IGF-1R in bone *in vivo*, although this was not demonstrated *in vitro* in this study. The osteoblast model may not be the best representative model for the actions of xenoestrogens on bone tissue within the milieu of endocrine signals *in vivo*.

Further research to distinguish the roles of the multiple estrogen receptors and IGF-1R, including their down-regulation at bone tissue and in the GH-IGF-1 hypothalamo-hypophyseal-hepatic-bone axes (Holloway and Leatherland 1998), may clarify IGF-1 disruption from exogenous estrogen exposure. Future studies should also investigate potential disruption of other growth factors involved in osteogenesis to target mechanisms and endpoints in vertebrate bone development sensitive to environmental contaminants.

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Chapter 5: General Discussion

Endocrine disruption is a concept that was conceived relatively recently in toxicology, and due to its potential ramifications on human and wildlife biology has gained credence not only within the scientific community, but within the public arena as well. A search on the popular internet search engine Google™ for "endocrine disruption" results in over 1.75 million references, the first of which is U.S. Environmental Protection Agency, the second of which is National Resource Defense Council, and the third of which is www.OurStolenFuture.org. *Our Stolen Future* is a book first published in 1996 that popularized the theories and scientific research devoted to endocrine disruption. Today, OurStolenFuture.org tracks popular and scientific literature regarding endocrine disruption studies, and suggests that as a result of the increasing number of chemicals linked to endocrine endpoints, increasing understanding of what those endpoints are, and the continuously lower doses or concentrations reported to induce changes in endocrine systems, there is no human being born within the last three decades "without some exposure in the womb. Every person has several hundred novel chemicals in their body, chemicals not part of human body chemistry before the 20th century."

Whether one considers this an alarmist, conservative, or realistic view, the fact remains that some chemicals documented in surface waters have the potential to disrupt homeostatic physiological functions within the body. The focus of this research was the perturbation of an endocrine signal involved in bone formation during development. The specific goals of this work included (a) developing an animal model to study both morphological and biochemical aspects of bone dysmorphogenesis, (b) observing that morphologogical endpoint in repeatable experiments with compounds both ubiquitous in the environment and bioavailable to an organism, and (c) identifying a biochemical marker within the endocrine system that may be linked to the morphological endpoint studied. These research objectives were carried out with experiments designed to conform to endocrine disruptor study parameters suggested by a National Toxicology Program Report (Melnick et al. 2002), namely use of an animal model responsive to endocrine-active agents of concern, husbandry of the model to reduce stress and encourage inter-animal variability, investigations into mechanistic biomarkers

of an observable effect, inclusion of long-term ramifications of early exposures, and the identification of windows of susceptibility and inclusion in exposures. Experimental design in some cases also entailed inclusion of low concentrations (sub-physiological levels) to high concentrations (supraphysiological and in some cases above environmentally relevant levels) to gauge concentration-dependent effects within a wide range of exposures. This aspect of the experimental design allowed determination of both low-dose effects and non-monotonic dose-response discussed in Chapter 1.

The hypothesis for this work is that estrogenic compound exposure in early development of an organism disrupts bone formation through perturbation of a growth-related endocrine signal, insulin-like growth factor-1 (IGF-1). Although a biochemical endpoint was the primary focus of this research, I initiated my investigations with bioassay development and range finding *in vivo*. I wanted to demonstrate the morphological endpoint of vertebral deformity from specific chemical exposure in the selected animal model before progressing to investigations into (biochemical) IGF-1 disruption. The sequence of scientific inquiry is therefore represented in this thesis.

A detailed description of experimental findings of each phase of research may be found within each chapter. In summary, the fathead minnow proved to be an excellent model for sublethal toxicity studies due to its high survival rates during and after the duration of chemical exposure. Fathead minnows are easily reared in the lab setting, are commonly and inexpensively available commercially, and allow for ease in microscopy studies at the juvenile stages examined in this work. Furthermore, they consistently demonstrated a low but detectable (≤4%) "background" incidence level of vertebral anomaly.

Fathead minnows were used in range finding and concentration-dependent effect studies with environmental estrogenic compounds, and demonstrated vertebral deformities in up to 61% of fish exposed to EE2, the synthetic estrogen used in birth control pills worldwide. Fish were affected in a non-monotonic concentration-response manner, most likely due to toxicity at the higher concentrations of EE2 used in the bioassays (100 µg/l), levels up to 100 times higher than the physiological estradiol level in fish and 1000 times higher than the level of any putative estrogen reported in surface waters. Deformities in 0.1 µg/l EE2-treated fish were significantly higher than for control fish, suggesting

either a lack of threshold proposed by Sheehan (2000), or a threshold not detectable in these studies. Preliminary range-finding assays with EE2 concentrations in the range of 0.001 to 10 µg/l at 3-10 days post-hatch resulted in deformity rates that were not higher than background rates in control fish. Further experiments with these lower concentrations for the 30-day exposure period as reported in Chapter 3 may clarify a threshold level. However, a compound with 1000 times lower affinity for the estrogen receptor than estradiol was used in bioassays (BPA), and was unable to induce vertebral anomalies above observed background levels.

The endocrine signal critical for normal bone development, IGF-1, was significantly reduced in fish exposed to EE2. These fish weighed less than their control counterparts, and had a less developed skeletal system based on the ranking system developed in early bioassays (discussed in detail in Chapter 2). IGF-1 significantly and positively correlated to fish development, and EE2-induced disruption of growth was evident in the bioassays. However, it is unclear whether weight reduction was a result of endocrine disruption within IGF-1 signaling pathways, or whether IGF-1 was reduced simply due to smaller fish size. The data does not therefore support the hypothesis of IGF-1 disruption from EE2. Experimental studies also included *in vitro* work with mouse osteoblasts to determine the direct effects of the estrogenic compounds on bone cells as compared to their effects *in vivo* in fish. Spectrophotometric and microscopic analysis of proliferation rates and immunoreactive protein levels (respectively) did not support the hypothesis that EE2 affects bone development solely at bone tissue, but more likely within the hypothalamo-hypophyseal-hepatic-bone axis. Further experiments to measure mRNA and protein expression of IGF-1 and receptors in the tissues within this axis may clarify this distinction, and contribute to the weight of evidence of endocrine disruption in vertebral development processes.

A recent workshop on fish aquaculture in Norway identified skeletal deformities as one of the most serious economic loss factors in farmed salmon, and emphasized the need for research to identify causes and risk factors (Waagbo et al. 2005). Implications for the results of the studies in this work are applicable not only to teleost fish swimming in estrogenic surface waters, but to any vertebrate exposed to potent estrogens. IGF-1 is highly conserved among

vertebrates with respect to structure, function, and regulation within the endocrine system, making all vertebrates susceptible to the ramifications of IGF-1 perturbation. It must be noted, however, that only supraphysiological (and high environmental) concentrations of a potent xenoestrogen induced the growth and morphological changes observed in the bioassays. However, the *in vitro* studies suggest possible effects at lower concentrations, and the pharmacological concept of additivity suggests multiple compounds with less potency than EE2, even when present in low concentrations, act in combination with each other or more potent estrogens to induce significant effects (Brian et al. 2005). The risk of vertebral dysmorphogenesis from environmental xenoestrogens in human and wildlife health is therefore not yet clear. The impacts of xenoestrogens, along with putative endocrine disruptors affecting multiple endpoints of human and wildlife health are still under investigation.

In 1996 the U.S. EPA formed the Endocrine Disruptor Screening and Testing Advisory Committee (EDSTAC), composed of industry, government, public health groups, worker safety groups, and academia representatives to make recommendations to EPA on how to develop the screening and testing program called for by Congress. According to the EDSTAC web site, the committee was "charged with developing consensus-based recommendations for a scientifically defensible screening program that would provide EPA the necessary information to make regulatory decisions about the endocrine effects of chemicals," decidedly a complicated task. EDSTAC finalized its projected goals of Priority Setting, Tier 1 Chemical Screening, and Tier 2 Chemical Testing in 1998. Currently the EPA is developing and validating methods for Tier 1 screening, with only a select few having yet undergone peer review. Once reviewed and implemented, the Tier 1 screening process will identify dozens of varied endpoints in endocrine signaling and thousands of compounds affecting those endpoints. Tier 2 testing will be a more refined and involved process of identifying specific risks from chemicals resulting in effects in Tier 1 screening at critical life stages from a wide range of doses through relevant exposure mechanisms. As a result of the expense of these aspects of the multigenerational Tier 2 testing, endpoints are limited to estrogen, androgen, or thyroid hormone systems. Although screening and testing does not currently include a vertebral morphology endpoint, more non-reproductive or non-thyroid endpoints may be

added as a result of the Tier 1 screening process, still under review. Furthermore, the low-dose studies incorporated in EPA's testing strategy may result in regulatory concentrations of estrogenic compounds in surface waters that are protective against bone dysmorphogenesis.

Perturbations of endocrine signals such as IGF-1 during critical stages of vertebral development are a source of concern. The incidences of disruptions in bone formation processes and of vertebral anomalies in humans and wildlife are discussed in Chapter 1. Depending on the parameters used to define human idiopathic scoliosis (typically the degree of curvature of the spine), the condition affects 3 to 8% of children worldwide. Most notably, the condition is commonly higher in girls than in boys, and worsens in severity as the child reaches adolescence (Robitaille et al. 1984). Girls are "eight times more likely to progress to a curve magnitude that requires treatment" than boys (National Scoliosis Foundation). In a 15-year study examining the prevalence of idiopathic scoliosis in Signapore schoolchildren, researchers found the prevalence rate increased in both boys and girls in increasing age groups, but the ratio of girls to boys significantly increased from 1.6 at 9-10 years of age to 6.4 at 11-12 years of age, the age of puberty onset (Wong et al. 2005). Also, the prevalence rate of scoliosis among girls within the 11-12 year age category significantly increased over the 15-year period of the study.

At the same time, more and more chemicals in the environment are detected (thanks in part to more sophisticated analytical techniques) that have some degree of putative endocrine disruption capability. While the role of EE2 in the multifactorial etiology of vertebral malformation in humans is unclear, the U.S. contraceptive market is estimated to grow at a steady pace each year. Endogenous estrogen signaling disruption from xenoestrogens may or may not have some role in the increased prevalence of scoliosis rates in children in recent decades. The increased amount of estrogenic signaling and the increase in severity of vertebral malformation during puberty may or may not have contributions from environmental compounds. What is clear is that future research to answer these questions is necessary.

Future studies incorporating both bioassay and molecular techniques may answer several questions regarding the etiology of vertebral dysmorphogenesis from xenoestrogens and other environmental contaminants. The reproducible endpoints of vertebral malformation and skeletal development disruption have

been established for the juvenile fathead minnow model. Multi-species comparisons at low (subphysiological) EE2 and other putative endocrine disrupting chemicals at and above levels found in the environment, along with appropriate additivity bioassays and modeling, would identify aquatic species susceptible to bone growth factor perturbation from contaminants in surface waters. Serum and tissue-specific protein levels may be obtained from larger fish species, although the development time is significantly longer in some species. Recent attempts to localize IGF-1, IGF-1 receptor (IGF-1R), and estrogen receptor proteins in whole fish sections using immunohistochemistry were unsuccessful due to auto-fluorescence of tissues; additional assays using non-fluorescent antibody labeling may be beneficial. The small size of 30 day post-hatch fathead minnows also made tissue processing difficult; longer exposures or the use of larger teleost models may be more appropriate in these investigations. Finally, the expanding science of proteomics may prove useful in identifying growth factors or proteins homeostatic bone development that may be disrupted by environmental compounds with endocrine-disrupting capabilities.

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