A FIELD STUDY OF METAL EFFECTS ON THE REPRODUCTIVE PHYSIOLOGY OF TELEOSTS

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Bachelor of Arts

Randolph-Macon Woman's College

Lynchburg, Virginia

1995

Submitted to the Faculty of the
Graduate College of the
Oklahoma State University
in partial fulfillment of
the requirements for
the Degree of
MASTER OF SCIENCE
August, 2001

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REPRODUCTIVE PHYSIOLOGY

OF TELEOSTS

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PREFACE

Increasing evidence indicates that the 70kDa stress protein family (HSP70) is important in a variety of physiological processes including protein chaperoning, steroidogenesis, protection against apoptosis, and general cellular stress responses in vertebrate organisms. The main objective of my study was to understand reproductive physiological effects of metals (cadmium, lead, and zinc) in female fishes with contrasting patterns of ovarian development. To accomplish this objective, a variety of subcellular, organ, and organismal responses were examined in wild fish populations inhabiting streams flowing through tailings piles remaining from commercial mining operations. Lead and Zinc mining spanned from the 1840s to the late 1960s in northeast Oklahoma and released potential endocrine modulating contaminants.

This project would not have been possible without the leadership, guidance, patience and support of my advisor, Dr. David M. Janz. I thank the individuals who assisted in field collections regardless of weather conditions (Dr. Janz, Dr. Lynn Weber, Sandra Brasfield, Matt TenEyck, Ruth Carlson, Mozhgan Savabiesfahani, Warren Coughlin, Anna Burrow, and Stephen Diamond). I would not have mastered the biochemical laboratory techniques necessary to complete my study objectives without the direction of Drs. Weber and Janz. I also express my appreciation to Dr. Roman Lanno of Department of Zoology and Dr. Veenstra of Department of Civil and Environmental Engineering for the use of their laboratory atomic absorption spectrometers. Karen

Bradham, Jason Conder, and Michael Krondak are tremendously appreciated for their assistance in understanding the analytical equipment.

I would also like to extend special appreciation to my family whose continued encouragement and support helped to make this goal a reality. And to my family away from home (Maral Kasparian, Sandy Brasfield, and Dr. Meredith Hamilton) whose love, laughter, and friendship has forever shaped my life.

Finally, I would like to thank my committee members; Dr. Dana Winkelman and Dr. Roman Lanno for their help and expertise in stream ecology and analytical chemistry. My research was funded by the Environmental Institute of Oklahoma State University. Many thanks to the Department of Zoology for providing me with a Teaching Assistantship and an opportunity to share scientific knowledge.

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Introduction

Reproduction is one of the most sensitive physiological functions disrupted in fish exposed to environmental stressors (Donaldson 1990). Environmental alterations at any stage of teleost gonadal development have the potential to impair reproductive capacity. A variety of environmental contaminants have been reported to impair the reproductive endocrine physiology of fish. In lab and field studies polychlorinated biphenyl (PCB) exposure reduces fertilization and hatching success, vitellogenin production, gonadal growth, milt production, and circulating reproductive steroids (Freeman and Idler 1975; Von Westernhagen et al. 1981; Spies and Rice 1988; Thomas 1988, 1990; Kidd et al. 1993). Similar reproductive responses have been induced by polycyclic aromatic hydrocarbon (PAH) exposure (Hose et al. 1981; Truscott et al. 1983; Johnson et al. 1988; Thomas 1988, 1989, 1990; Singh 1989; Monteiro et al. 2000), industrial discharges (e.g., pulp mill effluents) (Munkittrick et al. 1991; Van Der Kraak et al. 1992; Sandström 1996), and essential and nonessential heavy metals (e.g., lead, zinc, and cadmium) (Wright and Welbourn 1994; Baile and Kadu 1992; Victor et al. 1986; Thomas 1990, 1989; Brown et al. 1994). Long-term sublethal exposure to heavy metals, such as Cd, result in adverse effects on the physiological status of teleost fish and these effects have been shown to be mediated through alterations of endocrine function (Hontela 1998; Ricard et al. 1998).

Dietary and aqueous sources of heavy metal exposure have both been determined to be important routes of uptake in fish species (Williams and Giesy 1978; Harrison and Klaverkamp 1989; Bentley 1992; Farag et al. 1994, 1998). Primary target organs of metals accumulation vary from species to species (Kraal et al. 1995; Norey et al. 1990a),

as well as with route of exposure. Cadmium accumulation is greatest in the kidney, liver, and gill of catfish, fathead minnows, white sucker, rainbow trout, brook trout, roach, stone loach, and carp (Smith et al. 1976; Thomas et al. 1983, 1985; Kay et al. 1986; Brown et al. 1986; Giles 1988; Farag et al. 1994, 1998; Camusso et al. 1995;Thomann et al. 1997). Zinc concentrates in the gill, liver, kidney, and opercular bone and is homeostatically controlled by metallothionein protein complexes (Camusso et al. 1995). Lead accumulates primarily in the gill, spleen, kidney, and bone (Camusso et al. 1995).

Accumulation of essential and nonessential metals in tissues such as the kidney, liver, and gill is a result of sequestration of cations by low-molecular weight cysteine rich proteins known as metallothioneins (MT) (Farag et al. 1994). Metallotheioneins are expressed constitutively, always present, in cells for the maintenance of essential metal homeostasis within the organism during fluctuations caused by physiological processes (Olsson et al. 1987). Through induction, metallothioneins provide protection against nonessential metal toxicity in many species including teleosts (Thomas et al. 1983, 1985; Norey et al. 1990b; Suresh et al. 1993; Farag et al. 1995; Cinier et al. 1999). Metal toxicity of tissues results when concentration of metal exceeds the induction and binding capabilities of metallothionein proteins and excess metal becomes available to bind to macromolecules, such as cellular protein enzymes (Brown et al. 1990; Suresh et al. 1993). Brown et al. (1990) reported an equilibrium-dependent exchange of Cd between enzymatic and metallothionein pools of scorpionfish during chronic exposure.

Tissues such as gonads (den Besten et al. 1990; Thomas et al. 1985) and muscle (Cinier et al. 1999; Suresh et al. 1993) do not accumulate metals to the same degree as gill, kidney, and liver and this is proposed to be due to a lack of MT expression and

induction (den Besten et al. 1990). Because metallothioneins and metal binding proteins assist in the sequestration of essential and nonessential metals (Cousins 1979), it is possible that accumulated metals in tissues of low metallothionein induction will not be detoxified and result in toxicity (den Besten et al. 1990).

Several lab and field projects in fish have reported impairment of female reproductive capacity caused by metal exposure ranging from modification of endocrine function along the hypothalamus-pituitary-gonadal axis (Thomas 1989), to cell death in the ovary (Singh 1989) (Table 1). Since ovarian somatic cells are integral mediators of female reproductive function and are known to be sensitive targets of toxicants, my research focused on mechanisms of ovarian dysfunction in granulosa and theca cells.

During all stages of vertebrate oogenesis ovarian follicles lose their integrity and are eliminated before ovulation by the process of follicular atresia (Hsueh et al. 1994; Tilly 1996). Ovarian tissue atresia in fish can be induced by a number of natural and anthropogenic stressors (Saidapur 1978). Atresia may represent a significant determinant of the overall reproductive success of wild fish populations (Saidapur 1978; N'Da and Déniel 1993). The molecular mechanism responsible for ovarian follicular atresia is apoptosis (Tilly et al. 1991; Hughes and Gorospe 1991). The endocrine mediated apoptotic process plays a critical role in ovarian development and homeostasis (Kerr et al. 1972; Steller 1995; Hsueh et al. 1994; Tilly 1996). Apoptosis is considered an ongoing, normal event in the control of cell populations and occurs when cellular damage has exceeded a cells capacity to repair itself (Waalkes et al. 2000). Thus, in my study the degree of ovarian follicular cell apoptosis was measured as an indicator of atresia at the cellular level.

Table 1. Metal Effects on Teleost Reproductive Capacity

Metal	Effect	Reference
Cadmium	Oocyte maturation impaired and delayed ↓ Gonadosomatic Index	Baile and Kadu 1992; Brown et al. 1994; Singh 1989; Pereira 1993
	↓Vitellogenesis	Haux et al. 1988; Victor et al. 1986; Pereira 1993
	↓ Hepatosomatic Index	Pereira 1993
	Precocious ovarian growth ↑ovarian growth ↑vitellogenesis ↑steroidogenesis ↑gonadotropin secretion ↑egg production	Thomas 1990, 1989 Peterson et al. SETAC 21st Annual Meeting Abstract
	Hypothalamus-Pituitary-Gonadal axis disruption	Olsson et al. 1995
	↓ Vitellogenin transcription ↓ MT transcription	Singh 1989
	Ovary disorganization Inhibition of oogenesis Necrosis Fibrosis of lamellar walls ↓ Plasma Testosterone ↓ Plasma Estradiol	Spehar 1976
	Spawning and embryo production reduced	
Lead	↓ Ovarian growth ↓ Plasma Testosterone ↓ Plasma Estradiol	Thomas 1988
Zinc	Reduced egg production	Spehar 1976; Eaton 1973
	Chorion fragility	Holcombe et al. 1979
	Delayed sexual maturity	Pierson 1981

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Elevated apoptotic DNA fragmentation in association with increased expression of the 70kDa heat shock protein (HSP70) in ovarian follicles has also been attributed to contaminant exposure in wild fish populations (Janz et al. 1997, 2001). Heat shock proteins (HSPs) are highly conserved proteins found in all classes of organisms including bacteria, fungi, plants, invertebrates and vertebrates (Lindquist 1992). HSPs are vital components of cellular physiology as indicated by their presence in several intracellular locations (i.e. nucleus, cytoplasm, mitochondria, chloroplasts, endoplasmic reticulum) (Hightower 1991; Lindquist 1992; Feder and Hofmann 1999). In general, HSPs maintain cellular homeostasis and provide stress tolerance through regulation of protein-protein interactions (Craig and Gross 1991; Lindquist 1992).

The present study focused on the 70kDa family of stress proteins (HSP70). The HSP70 family is one of the largest families of stress proteins, and is expressed constitutively as well as induced in response to a wide range of biotic and abiotic stressors. HSP70 is found in most cells as a constitutive 73kDa form (hsc70) and an inducible 72kDa form (hsp72) (Feder and Hofmann 1999). Both the constitutive and inducible forms act to stabilize proteins in the process of maturation (Beckmann et al. 1990). Acting as molecular chaperones, HSP70s interact transiently with translating polypeptides to assist in the correct folding and assembly of proteins into physiologically active conformations, as well as escorting proteins to correct locations in the interior and exterior of the cell (Beckmann et al. 1990; Craig and Gross 1991).

Induction of HSP70 occurs rapidly upon proteotoxic stress, within minutes of temperature elevation (Morimoto et al. 1992) and within two hours following Cd exposure (Levinson et al. 1980). Proteotoxicity is the disruption of protein structure and

function resulting in cellular accumulation of unfolded and aggregated proteins (Hightower 1991). The inducible form of HSP70 is expressed in response to proteotoxic factors such as temperature fluctuations, hypoxia, metals, pesticides, and complex mixtures of organic contaminants (Ryan and Hightower 1994; Williams et al. 1996; Janz et al. 1997, 2001; Aït-Aïssa et al. 2000; De Smet and Blust 2001; reviewed in Feder and Hofmann 1999). The rapid induction of HSP70 in response to sublethal concentrations of contaminants has led them to be proposed as sensitive indicators of stress at the cellular level before toxicity (Sanders 1993; Feder and Hofmann 1999).

HSP70s have been reported to protect cells against apoptosis in vertebrate organisms by affecting signaling pathways in the cascade of physiological signals resulting from stress-induced apoptosis (Samali and Cotter 1996; Mosser et al. 1997; Buzzard et al. 1998; Kwak et al. 1998; Mallouk et al. 1999). Janz et al. (1997, 2001) reported correlations between increased expression of HSP70 and elevated ovarian cell apoptosis in white sucker (*Catostomus commersoni*) chronically exposed to bleached kraft pulp mill effluent. Dix (1997) reports that induction of HSP70 protects mammalian embryos from toxicant exposure effects, thus being a useful indicator of reproductive impairment due to contaminant exposure. In my study expression of HSP70 in ovarian tissue and ovarian follicular apoptosis were measured in female fish chronically exposed to metals.

In vivo and *in vitro* studies have demonstrated impacts of environmental stressors on ovarian steroid biosynthetic capacity (McMaster et al. 1995). Alterations in circulating sex steroid concentrations (testosterone and 17β-estradiol) have been observed in teleost species exposed to various toxicants (Munkittrick et al. 1991; McMaster et al. 1995,

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1996a; Monteiro 2000), including essential and nonessential metals (Thomas 1988, 1989, 1990). As part of their constitutive responsibilities, HSP70s regulate steroid biosynthesis through mediation of receptor-ligand binding (Welch 1993). The measurement of circulating levels of gonadal steroid hormones has been demonstrated as a useful tool for evaluating the reproductive impacts of xenobiotic contaminants at the organism level (McMaster et al. 1992). Thus, in my study serum concentrations of testosterone and 17β-estradiol were quantified as indications of reproductive endocrine function and homeostasis.

Research objective and hypotheses

Knowledge of HSP70 function in response to exogenous stressors in fish species is primarily based upon research conducted using *in vitro* techniques such as cell lines and primary cultures of fish cells (Iwama et al. 1998). Fewer investigations have been conducted in wild populations of fish species chronically exposed to xenobiotics or subjected to natural temperature extremes. This research compared HSP70 expression in gill, liver, ovary, and kidney in two fish species inhabiting a metal contaminated stream and a reference stream within the same watershed of northeastern Oklahoma. My research examined tissue specific expression of HSP70 in response to a natural stressor (seasonal temperature fluctuation) and chronic contaminant stressors (elevated Cd, Pb, Zn). I used females of two fish species inhabiting different niches of the stream to examine potential routes of contaminant exposure. Also, I collected two species that differ in reproductive strategy in order to examine reproductive responses to metal exposure in fish with differences in ovarian development. I hypothesized that there would be temperature-, toxicant-, and species-related differences in expression of HSP70

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and reproductive parameters. To test these hypotheses, HSP70 protein expression, steroidogenesis, ovarian follicular apoptosis, and organismal responses were determined in black bullhead (*Ameiurus melas*), a benthic species that spawns only once throughout the breeding season (synchronous), and bluegill sunfish (*Lepomis macrochirus*), a pelagic species that spawns multiple times throughout the breeding season (asynchronous), collected in winter (teleost recrudesence) and spring (pre-spawning).

My overall objective was to provide insight into the relationship between stress at the cellular/ tissue level (HSP70 expression/ ovarian follicular cell apoptosis) and physiological condition of the whole organism (steroidogenesis, condition factor, gonadosomatic and hepatosomatic indices). The following hypotheses were formed based on studies correlating fish reproductive impairment with environmental toxicant exposure (Van Der Kraak et al. 1992; McMaster et al. 1996b; Janz et al. 1997).

1. Female fish inhabiting a metal (Cd, Pb, Zn) contaminated stream will exhibit increased ovarian follicular cell apoptotic cell death compared to fish inhabiting an uncontaminated stream.
2. Female fish inhabiting a metal contaminated stream will exhibit suppressed steroidogenesis compared to fish inhabiting an uncontaminated stream.
3. Condition factor, gonadosomatic index (GSI), and hepatosomatic index (HSI), will be suppressed in fish chronically exposed to metals contamination compared to fish inhabiting an uncontaminated stream.
4. HSP70 expression will be higher in kidney, liver, gill, and ovarian tissues in wild female fish chronically exposed to metals compared to fish inhabiting an uncontaminated stream.

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5. HSP70 expression will be elevated in kidney, liver, gill, and ovarian tissues collected during spring compared to tissues collected during the winter.

Material and Methods

Study Site

The study stream (Tar Creek) flows through one of the original EPA Superfund sites known as the Tri-State Mining Area, located in northeastern Oklahoma. Tar Creek flows through tailings piles remaining from commercial lead and zinc mining operations that spanned from the 1840s to the late 1960s in northeast Oklahoma. A previous study reported levels of dissolved Cd, Pb, and Zn in Tar Creek (26μg/L, 36μg/L, and 7,786μg/L, respectively) (U.S. EPA 1994) that exceed the National Recommended Water Quality Criteria established for chronic exposures to aquatic organisms (Cd 2.2μg/L, Pb 2.5μg/L, Zn 120μg/L) (U.S. EPA 1998). A stream in the same watershed northeast of Tar Creek (Lytle Creek) is not impacted by tailings runoff or mining discharges and served as the reference stream.

Fish collection and sampling

Two fish species with contrasting niches were collected from a 300meter stretch of stream at each site by electrofishing and angling. Female black bullhead (*Ameiurus melas*), a stream bottom feeder and synchronous spawner, and female bluegill sunfish (*Lepomis macrochirus*), a water column feeder and asynchronous spawner, were collected in May-June 1999 and 2000 (pre-spawning period) and February 2000 (recrudescence period) in order to understand potential effects of metals at different stages of ovarian development. Only adult (age 2+, as confirmed by otolith analysis) fish were used. Fish were weighed and fork lengths were measured (Appendix A). Blood

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was taken from the caudal artery using a heparinized syringe and stored at 4°C overnight before centrifugation (10 min, 2940 x g and 4°C) and serum collection. Serum was stored at -20°C until analysis. Liver, kidney, ovary and gill were excised from fish and immediately frozen in liquid nitrogen. Condition factors ((body weight/length^3) x 10^5), gonadosomatic (gonad weight/(body weight - gonad weight) x 100), and hepatosomatic indices (liver weight/(body weight - liver weight) x 100) were calculated from individual fish.

Tissue homogenization and Western immunoblots

HSP70 expression in liver, kidney, ovary and gill tissue was detected and quantified using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), Western immunoblotting, and optical densitometry of immunoreactive bands (Janz et al. 1997). A crude protein homogenate was prepared from 50-75 mg of tissue placed in 125μl ice-cold homogenization buffer (50 mM HEPES, 150 mM NaCl, 1 mM EGTA, 1.5 mM $MgCl_2$, 1% (v/v) Triton X-100, 10% (v/v) glycerol, pH7.5) containing protease inhibitors (0.1mg/ml AEBSF, 20 μg/ml soybean trypsin inhibitor, and 1.9μg/ml aprotinin). Tissues were homogenized on ice 5 x 10 sec at 3000 rpm using a 1ml glass-teflon Potter-Elvehjem homogenizer. Homogenate was transferred to a 1.5ml microcentrifuge tube, along with 2 x 62.5 μl washings of the homogenization tube and rotated for 1 hr at 4°C. Samples were centrifuged for 20 min at 10,000 x g and 4°C, and the resulting supernatant was stored at -80°C for use in Western blots. Protein was measured in the supernatant using a DC protein assay (BioRad, Hercules, CA). To remove large lipoproteins from

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ovary samples the supernatant was centrifuged overnight at 5000 x g and 4°C and filtered through 100kDa NMW Ultrafree-MC (Millipore, Bedford, MA) filters to isolate those proteins less than 100kDa.

Protein homogenate (150μg protein/lane: gill, liver, kidney; 20 μg protein/lane: ovary) was separated using SDS-PAGE with 10% acrylamide large format gels at 32 mA/gel and transferred to 0.45 μm nitrocellulose membranes (Biorad, Hercules, CA) at 30V for 16hr and 4°C. HSP70 was detected by immunoblotting using a monoclonal mouse anti-bovine HSP70 antibody at 1:5000 dilution (Sigma, St. Louis, MO). Blots were visualized with 1:2000 horseradish peroxidase-conjugated goat anti-mouse antibody (Santa Cruz Biotech, Santa Cruz, CA) and enhanced chemiluminescence (Pierce, Rockford, IL). The optical density of bands was determined using NIH Image software. To minimize variation in HSP70 quantification, all blotting and washing steps were performed identically and film was exposed to membranes for identical amounts of time, which was determined in preliminary experiments.

Hormone extraction and analysis

Enzyme-linked immunosorbent assay (ELISA) kits were used to determine serum concentrations of testosterone (Cayman Chemical, Ann Arbor, MI) and 17β-estradiol (Oxford Biomedical Research, Inc., Oxford, MI) following ether extraction of the steroids from serum media (percent extraction efficiencies mean ± SEM, n=2; 81.5 ± 8.8 estradiol, 84.1 ± 2.8 testosterone). Ether extractions function to free steroid molecules from their binding plasma proteins (McMaster et al. 1992). Following ether extraction, samples were reconstituted in radioimmunoassay (RIA) buffer (40.49mM Na_2HPO_4, 8.17mM NaH_2PO_4, 0.001%(w/v) gelatin, pH7.6) and stored at -20°C until ELISA assays.

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Serum samples were assayed in duplicate when sample volume allowed. ELISA validation was performed for bullhead for both hormones measured, while limited sunfish samples only allowed for partial validation. Interassay coefficients of variation, a measure of variability between assays, were 40% and intra-assay coefficients of variation, a measure of pipetting variability within a single assay, were <15% for testosterone in black bullhead. Intra-assay coefficients of variation were <10% for testosterone in bluegill sunfish. Intra-assay coefficients of variation were <15% for 17β-estradiol measured in bullhead. Parallelism between serial dilutions of teleost serum and standard hormone solutions was demonstrated for both species, confirming that the ELISA kits were sensitive to the study species examined in the present study (Appendix B).

<u>DNA isolation and analysis</u>

Total genomic DNA from ovarian follicles was extracted and phenol/chloroform-purified (Janz and Van Der Kraak 1997). DNA was isolated from 20-30mg of ovarian tissue, quantified by absorbance at 260nm, and stored at -20°C. The yield of DNA from 24.96 ± 0.65mg (mean ± SEM; n=21) of ovarian tissue was 4.54 ± 0.73 µg which ranged in purity from 92-100% (97.95 ± 0.37%). The isolated DNA was 3'-end labeled using the method described by Janz and Van Der Kraak (1997) with slight modifications. Briefly, 2 µg DNA was labeled in 50 µl total reaction volume containing 0.2M potassium cacodylate, 25nM Tris-HCl (pH=6.6), 2.5 mM $CoCl_2$, 25 U terminal transferase enzyme, and 0.26 µM [α^{32}P]-dideoxyATP (1500Ci/mmol; Amersham, Pharmacia Biotech, Piscataway, NJ). Following agarose gel electrophoresis, autoradiography film was exposed to dried gels in plastic wrap for 36-48 hours at -80°C. Low molecular weight DNA (<15kb determined

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using the M.W. standard) was cut from each lane and counted using liquid scintillation to quantify the extent of apoptotic DNA fragmentation (Tilly and Hsueh 1993).

Physicochemical parameters

Physicochemical parameters (pH, conductivity, hardness, alkalinity) were measured in water samples collected in triplicate from each stream each sampling season. PH of water samples was determined with samples at room temperature using EPA method 150.1 (U.S. EPA 1979) and the Labomatic Model 165 pH/mV electrometer (Instrumentation Laboratory, Inc., Boston, MA). Conductivity was measured with an Orion conductivity meter Model 126 (Orion, Cambridge, MA) using EPA method 120.1 (U.S. EPA 1979) and reported as μS/cm. Total hardness was measured using the titration method, EPA method 130.2 (U.S.EPA 1979), with titration and indicator reagents from HACH Company (Loveland, CO) and reported as mg/L $CaCO_3$. Alkalinity was measured using EPA method 310.1 (U.S. EPA 1979) and 0.020 N sulfuric acid standard solution (HACH Company, Loveland, CO) and reported as mg/L $CaCO_3$.

Water and liver sample preparation and metals analysis

Cd, Pb, and Zn were analyzed in the dissolved and suspended portions of water samples collected in triplicate within the same fish collection vicinity of each stream using flame (Cd, Zn) (FAA) and graphite furnace (Pb) (HGA) atomic absorption spectrometry. Following field collection water samples were transported to the laboratory on ice to be filtered using a 0.45-μm-pore-diameter cellulose acetate membrane within 24 hrs of collection. Samples were preserved by acidifying with concentrated metals grade HNO_3 (Fisher Scientific, Pittsburgh, PA) to pH 2-4 and stored at 4°C until preparation for analysis of dissolved metals. Method detection limits are the lowest reliable

measurement for the equipment and method and were determined as lowest observed quantitation (LOQ) by multiplying the standard deviation of absorbance from seven method blanks by a factor of 10. The dissolved concentrations of Zn were determined by flame atomic absorption spectrometry (FAA) (Zn wavelength 213.9 nm and LOQ 7 μg/L) directly. Dissolved portions of the samples were concentrated 1:500 by boiling and reconstitution with 0.5 N HNO_3 for Cd detection by FAA (Cd wavelength 228.8 nm and LOQ 28 μg/L). To determine suspended metals in the water samples, the filters from the above described filtration were digested in 30% metals grade HNO_3 at 76°C for 48-72 hrs to release substrate bound metals into solution and reconstituted to 150 ml with 0.5 N HNO_3. Samples were concentrated 1:30 for Cd analysis by FAA (Cd wavelength 228.8 nm and LOQ 28 μg/L) and measured directly for Zn concentrations by FAA (Zn wavelength 213.9 nm and LOQ 7 μg/L). Graphite furnace spectrometry (HGA) was used to detect Pb (wavelength 283.3 nm and LOQ 2.5 μg/L) in 1:50 concentrated dissolved and 1:30 concentrated suspended portions of water samples diluted with 0.5N metals grade HNO_3 accordingly.

Concentrations of metals were determined in liver (0.5g –1.0g) samples prepared for analysis using a combined concentrated metals grade HNO_3 and heat wet-tissue digestions (U.S. EPA 1991). Samples were reconstituted in 0.5 N HNO_3 and analyzed by FAA (Zn wavelength 213.9 nm and LOQ 7 μg/L) or graphite furnace (Cd wavelength 228.8 nm and LOQ 0.08 μg/L; Pb wavelength 283.3 nm and LOQ 2.5 μg/L) atomic absorption spectrometry.

Statistics

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Results are expressed as mean ± SEM and numbers (n) indicate number of fish sampled. Student *t*-tests were used to detect differences between the biochemical parameters measured in reference and metal exposed fish and the physicochemical parameters measured in each stream. Spring collection data sets were pooled when sample means were not significantly different. A $p<0.05$ was considered to be statistically significant.

Results

Physicochemical parameters

Physicochemical parameters in Tar Creek (pH, hardness, alkalinity, and conductivity) were significantly elevated in comparison to Lytle Creek (Table 2) regardless of collection season. Flame and graphite furnace atomic absorption spectrometry of water samples determined Cd, Pb, and Zn concentrations in the suspended portions of water samples collected from Tar Creek to be significantly higher than metal concentrations in suspended portion samples from Lytle Creek (p=0.005, 0.02, 0.009, respectively; n=5-6; Figure 1). Cd and Zn were significantly higher in the dissolved portions collected from Tar Creek in comparison to Lytle Creek samples (p=0.005, Figure 1A; $p<0.0001$; n=5-6; Figure 1C).

Metal concentrations in liver samples

Bluegill sunfish and black bullhead collected from Tar Creek (study site) accumulated significantly elevated levels of Cd and Zn in liver tissue during both spring and winter collections (Cd: Figure 2, Spring 1999 (bluegill sunfish p=0.01; n=4-10) (black bullhead p=0.001; n=4-6), Winter 2000 (bluegill sunfish p=0.002; n=5-6) (black bullhead p=0.01; n=2-3); Zn: Figure 4, Spring 1999 (bluegill sunfish p=0.03; n=4-10) (black bullhead p=0.004; n=3-6), Winter 2000 (bluegill sunfish $p<0.0001$; n=5-6) (black bullhead

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p=0.0006; n=2-3)) in comparison to liver samples of Lytle Creek (reference site) fish. Pb accumulated significantly in the livers of bluegill sunfish collected from Tar Creek during spring 1999 (p=0.05; Figure 3; n=5-6).

HSP70 expression

The anti-HSP70 antibody used in this study recognizes both the constitutive 73kDa and inducible 72kDa forms of HSP70. Although I observed band doublets at 70kDa on large format SDS-PAGE gels, I was not able to distinguish between constitutive and inducible forms in either species, and thus total HSP70 was quantified using densitometry of both immunoreactive bands present at 70kDa. HSP70 expression in gill, liver, kidney and ovary are shown separately for spring 1999 (Figure 5) and spring 2000 (Figure 6). During the spring 1999 collections (water temperature 26.1 ± 1.1 °C) expression of HSP70 was significantly elevated in both liver and kidney tissue of bluegill sunfish inhabiting the metal contaminated stream (Tar Creek) (p=0.039 and p=0.042, respectively; n=4-10 Figure 5A). Expression of HSP70 in ovary and gill tissues collected from bluegill inhabiting Tar Creek (p=0.09 and, p=0.078, respectively; Figure 5A) were not statistically significant. No statistically significant differences in HSP70 were observed between study sites in gill, kidney, liver or ovary tissue of black bullhead collected spring 1999 (Figure 5B).

During the subsequent spring 2000 collection (water temperature 27.5 ± 2.5 °C), HSP70 expression was significantly elevated in kidney tissue collected from black bullhead chronically exposed to metals (p=0.026; n=5-9 Figure 6B), but no difference was determined in bluegill sunfish (p=0.242; n=9-10 Figure 6A). In contrast to the previous spring collection, HSP70 expression was significantly elevated in ovarian tissue

	Lytle Creek			Tar Creek		
	Spring 1999	Winter 2000	Spring 2000	Spring 1999	Winter 2000	Spring 2000
Water temp (°C)	26.7 ± 1.8	5.6	30.0	25.6 ± 1.5	4.0	25.0
pH	7.02 ± 0.04	7.17 ± 0.03	7.38 ± 0.02	*7.47 ± 0.04	*7.47 ± 0.03	*7.60 ± 0.003
Hardness (as mg/L $CaCO_3$)	83.5 ± 0.96	306.67 ± 6.57	138.27 ± 0.13	*178.29 ± 15.29	*652.00 ± 52.62	*416.67 ± 1.33
Alkalinity (as mg/L $CaCO_3$)	47.11 ± 2.35	47.33 ± 1.33	98.67 ± 0.67	*68.57 ± 4.84	*88.00 ± 2.31	*122.03 ± 0.03
Conductivity (uS/cm)	198.51 ± 6.69	673.03 ± 0.03	309.67 ± 0.67	*459.14 ± 35.18	*1393.0 ± 0.03	*1095.0 ± 2.08

Table 2. Physicochemical parameters measured from water samples collected from Tar Creek (study site; n=7 (1999), n=3(2000)) or Lytle Creek (reference site; n=7 (1999), n=3(2000)) in triplicate each season. * An asterisk indicates Tar Creek samples are significantly different from Lytle Creek ($p<0.05$).

Oklahoma State University

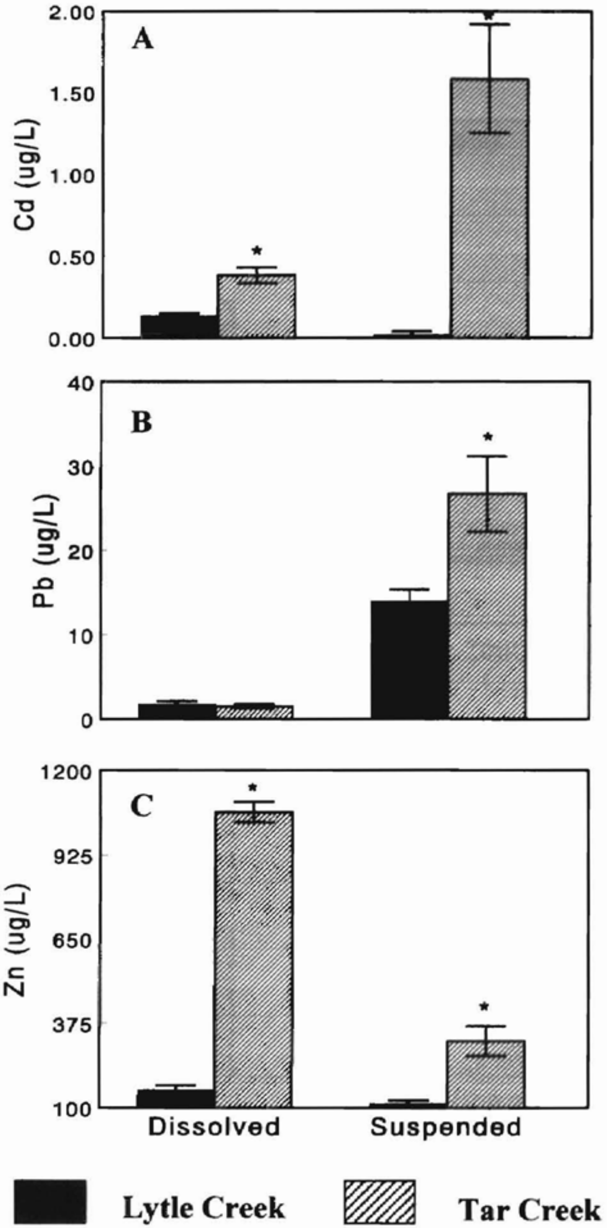

Figure 1. Concentrations (mean ± SEM) of cadmium (A), lead (B) and zinc (C) (μg/L) measured by flame (Cd and Zn) and graphite furnace (Pb) atomic absorption spectrometry in the dissolved and suspended portions of water samples collected from Lytle Creek (reference site: solid bars) and Tar Creek (study site: hatched bars) n=5-6 spring 1999. *An asterisk indicates Tar Creek samples are significantly different from Lytle Creek ($p<0.05$).

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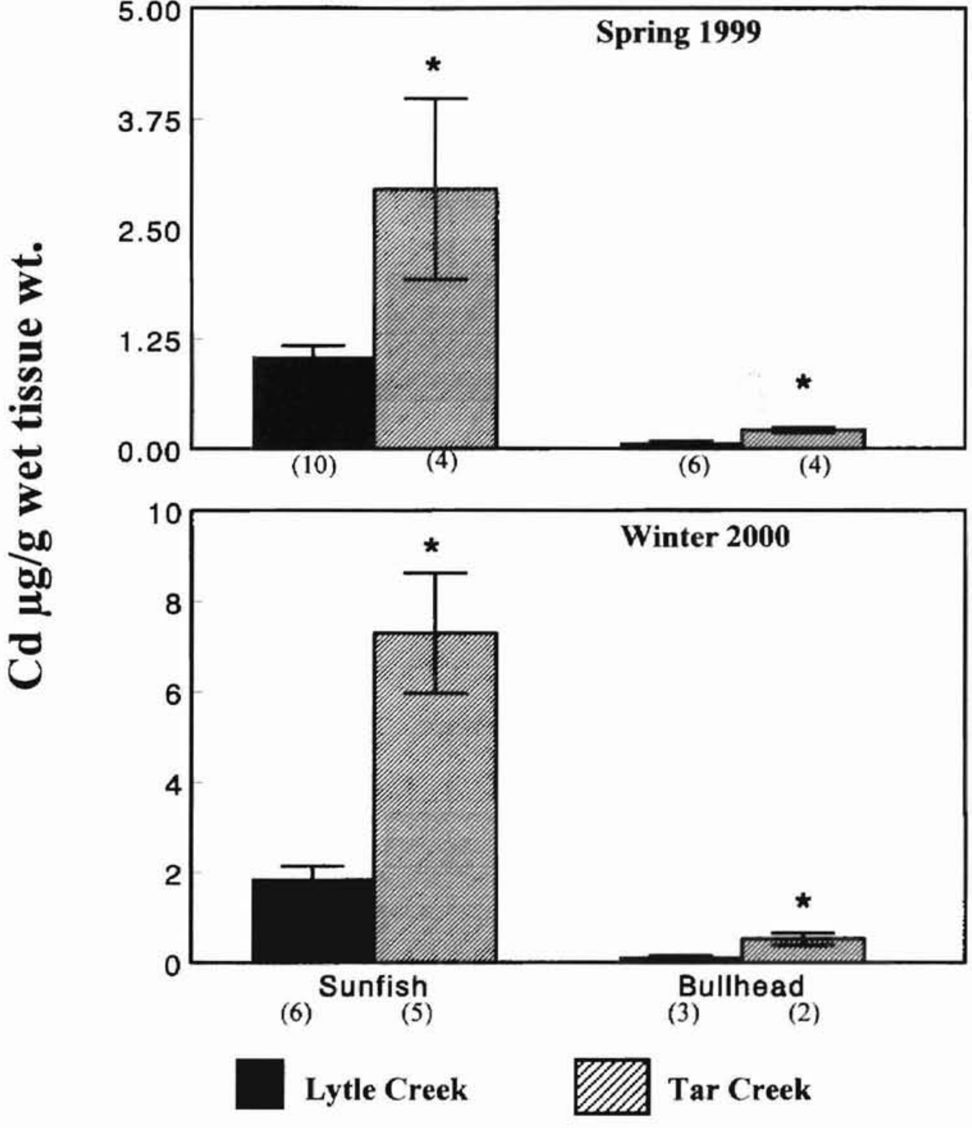

Figure 2. Concentrations (mean ± SEM) of cadmium determined by graphite furnace atomic absorption spectrometry in liver tissue (µg/g) of bluegill sunfish (*Lepomis macrochirus*) and black bullhead (*Ameiurus melas*) collected in Spring 1999 (top) and Winter 2000 (bottom) from Lytle Creek (reference site: solid bars) and Tar Creek (study site: hatched bars) (n) * An asterisk indicates Tar Creek samples are significantly different from Lytle Creek (p<0.05).

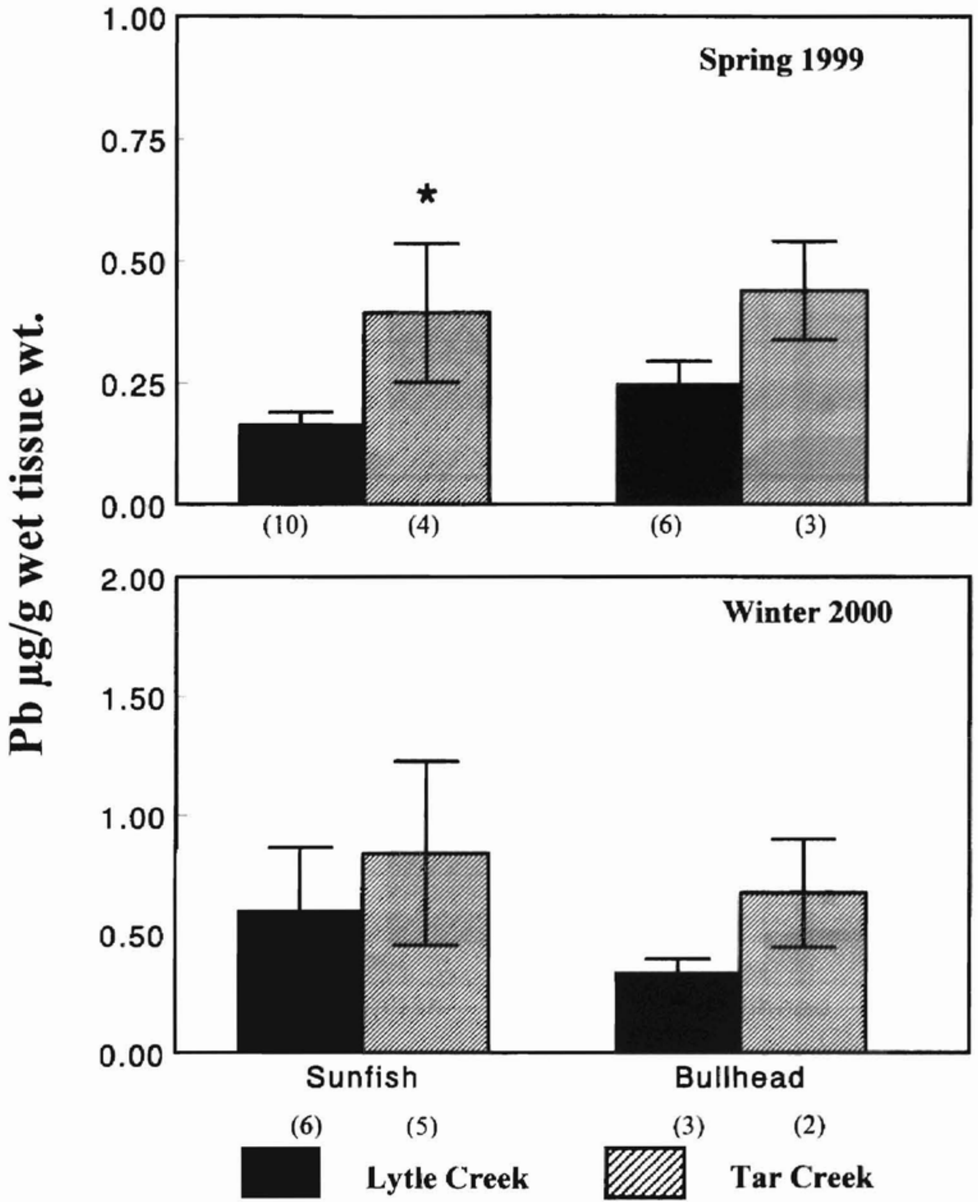

Figure 3. Concentrations (mean ± SEM) of lead determined by graphite furnace atomic absorption spectrometry in liver tissue (µg/g) of bluegill sunfish (*Lepomis macrochirus*) and black bullhead (*Ameiurus melas*) collected in Spring 1999 (top) and Winter 2000 (bottom) from Lytle Creek (reference site: solid bars) and Tar Creek (study site: hatched bars) (n). * An asterisk indicates Tar Creek samples are significantly different from Lytle Creek ($p<0.05$).

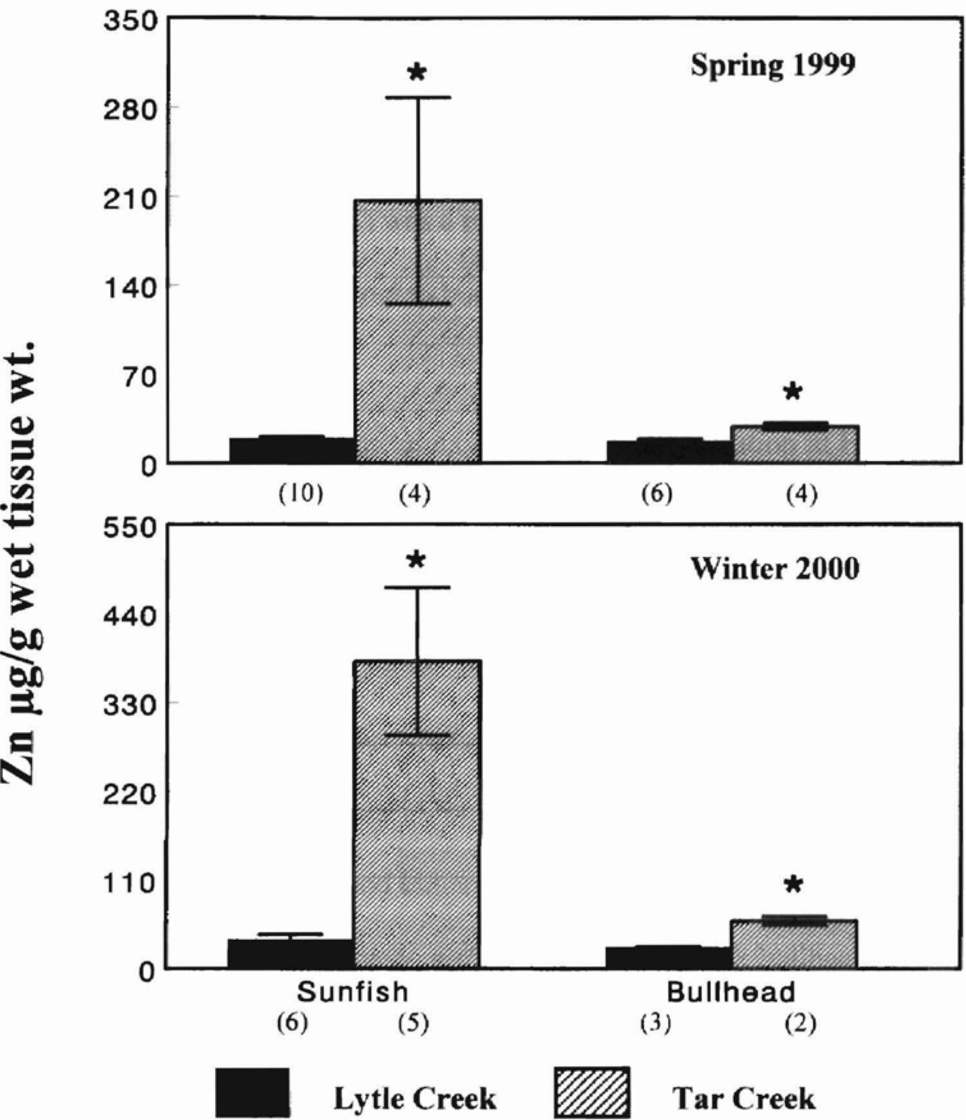

Figure 4. Concentrations (mean ± SEM) of zinc determined by flame atomic absorption spectrometry in liver tissue (µg/g) of bluegill sunfish (*Lepomis macrochirus*) and black bullhead (*Ameiurus melas*) collected in Spring 1999 (top) and Winter 2000 (bottom) from Lytle Creek (reference site: solid bars) and Tar Creek (study site: hatched bars) (n). * An asterisk indicates Tar Creek samples are significantly different from Lytle Creek (p<0.05).

of bluegill collected from Tar Creek (p=0.008; Figure 6A), but not in black bullhead. No differences were detected between sites in levels of HSP70 expression in gill or liver collected from either species in spring 2000 (Figure 6A & B).

The only significant difference observed in HSP70 expression in winter 2000 (water temperature 4.8 ± 0.80 °C) was a reduction in HSP70 in the liver of black bullhead collected from Tar Creek compared to bullhead collected from Lytle Creek ($p = 0.019$; n=2-3,Figure 7B).

To exclude the effect of metal exposure, seasonal variation in HSP70 expression was determined by comparing HSP70 levels in gill, liver, kidney and ovary from bluegill sunfish and black bullhead inhabiting the reference site (Lytle Creek) during spring and winter. Because the means of separate spring collections were not significantly different data sets were pooled for all tissues except ovarian tissue collected from bluegill sunfish. There was no significant difference in the expression of HSP70 between spring and winter collections in gill, liver, and kidney tissue of both species (bluegill sunfish: p=0.46, p=0.31, p=0.10, respectively, n=6-19 Figure 8A; black bullhead: p=1.0, p=0.06, p=0.21, respectively, n=3-11 Figure 8B). HSP70 expression was significantly elevated in ovarian tissue of black bullhead collected during spring in comparison to winter (p=0.02; n=3-11; Figure 8B). In contrast to HSP70 expression was elevated in ovary tissue collected from bluegill in winter compared to spring 1999 but not different from spring 2000 measurements (p=0.03; n=6-10; Figure 8A).

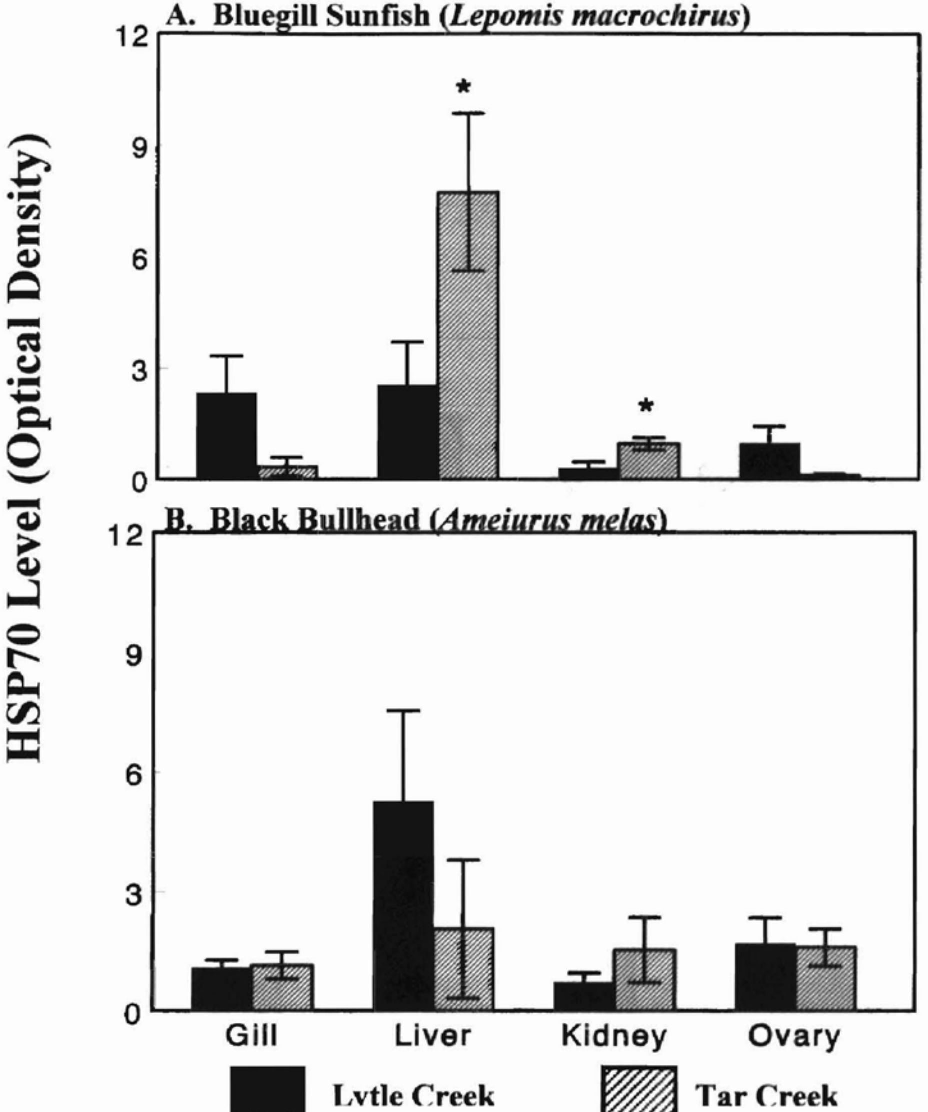

Figure 5. Expression of the 70kDa heat shock protein (HSP70) measured as optical density (mean ± SEM) in gill, liver, kidney and ovary of bluegill sunfish (*Lepomis macrochirus*) (A) and black bullhead (*Ameiurus melas*) (B) collected from Lytle Creek (reference site: solid bars: n=10 (A), n=6(B)) and Tar Creek (study site: hatched bars: n=4) during spring 1999 (pre-spawning: water temperature 26.1 ± 1.1°C). * An asterisk indicates Tar Creek samples are significantly different from Lytle Creek ($p<0.05$).

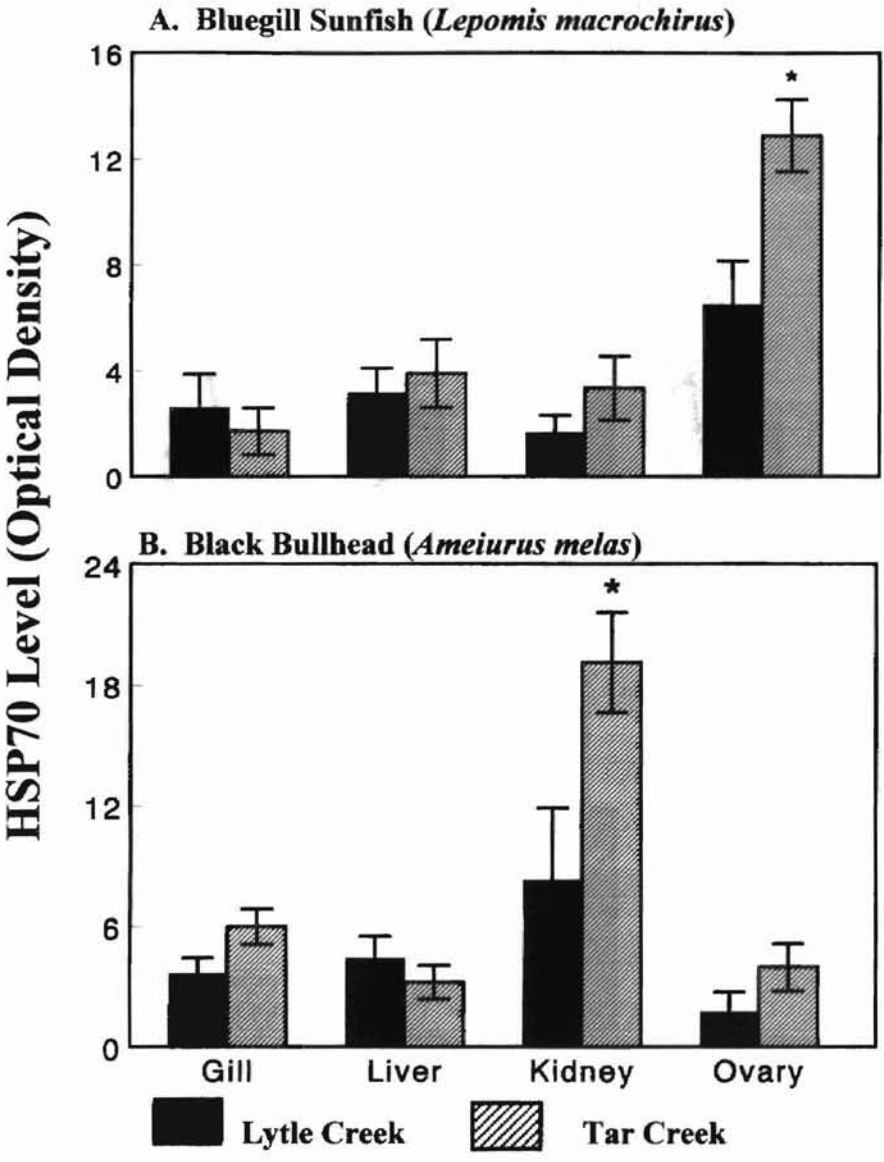

Figure 6. Expression of the 70kDa heat shock protein (HSP70) measured as optical density (mean ± SEM) in gill, liver, kidney and ovary of bluegill sunfish (*Lepomis macrochirus*) (A) and black bullhead (*Ameiurus melas*) (B) collected from Lytle Creek (reference site: solid bars: n=9(A), n=5(B)) and Tar Creek (study site: hatched bars: n=10(A), n=9(B)) during spring 2000 (pre-spawning: water temperature 27.5 ± 2.5°C). * An asterisk indicates Tar Creek samples are significantly different from Lytle Creek (p<0.05).

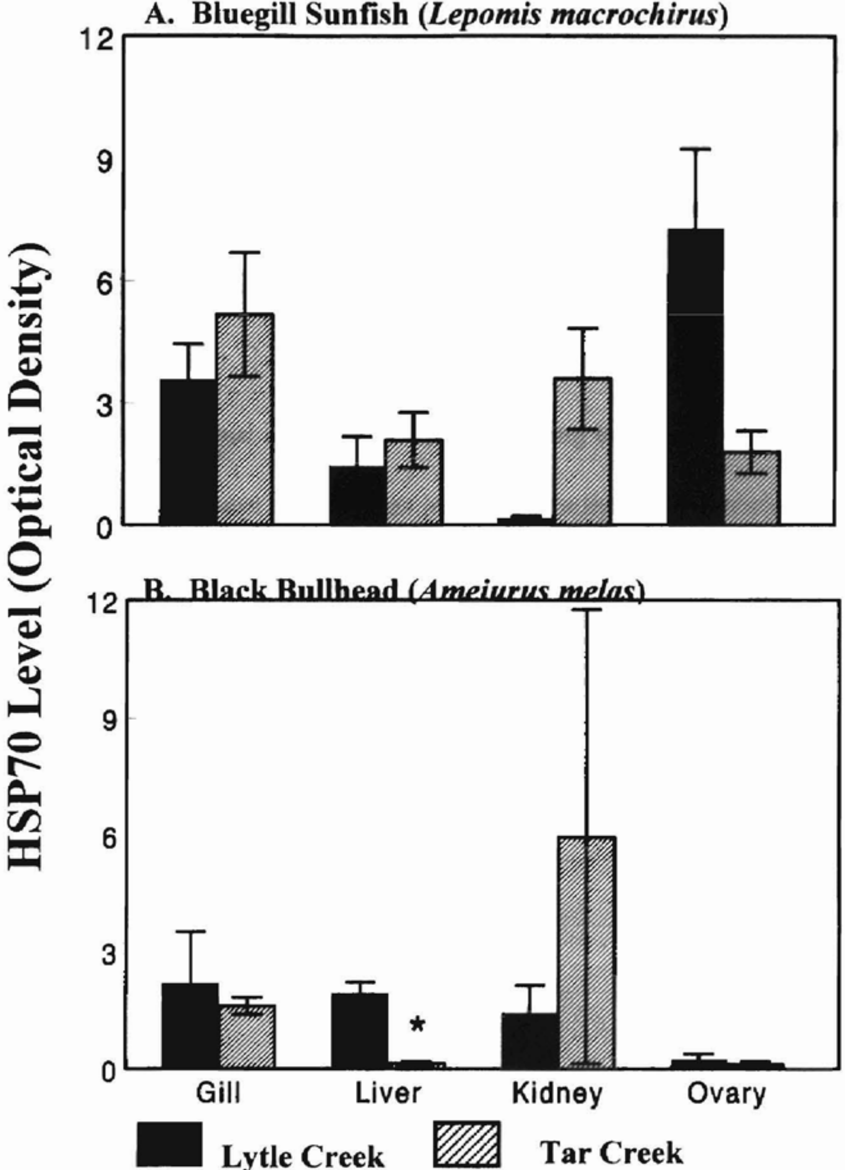

Figure 7. Expression of the 70kDa heat shock protein (HSP70) measured as optical density (mean ± SEM) in gill, liver, kidney and ovary of bluegill sunfish (*Lepomis macrochirus*) (A) and black bullhead (*Ameiurus melas*) (B) collected from Lytle Creek (reference site: solid bars: n=6(A), n=3(B)) and Tar Creek (study site: hatched bars: n=6(A), n=2(B)) during winter 2000 (recrudescence: water temperature 4.8 ± 0.80°C). * An asterisk indicates Tar Creek samples are significantly different from Lytle Creek ($p<0.05$).

Steroidogenesis

Circulating levels of 17β-estradiol and testosterone were determined from serum ether extractions using ELISA analysis. Testosterone was significantly reduced in bluegill sunfish collected from Tar Creek during recrudescence (p=0.04; n=3-4; Figure 9B), however there was no difference determined between sites during the pre-spawning collection (Figure 9B). No differences were determined between sites in levels of circulating 17β–estradiol in bluegill sunfish during either collection season (Figure 9A). There were no significant differences in 17β-estradiol and testosterone concentrations in black bullhead chronically exposed to metals (Tar Creek) collected during the pre-spawning period of teleost ovarian development (p=0.10; n=5-7; p=0.20; n=11-12; respectively; Figure 10). No differences existed between sites in hormone concentrations determined in black bullhead collected during the recrudescent period of ovarian development (Figure 10).

Apoptosis

DNA was isolated from ovarian granulosa and theca cells collected during the pre-spawning period of ovarian development of both species and the 3'-ends were radiolabeled [^{32}P] and separated by gel electrophoresis to determine the extent of DNA fragmentation (a sensitive indicator of apoptosis). No significant difference in ovarian follicular cell apoptosis was observed in either species inhabiting the metal contaminated stream (Tar Creek) (bluegill sunfish p=0.37; black bullhead p=0.08; n=5-6; Figure 11).

Organismal Responses

As measures of stress at the organismal level, condition factors and gonadosomatic (GSI) and hepatosomatic indices (HSI) were calculated (Table 3). Values for spring collections

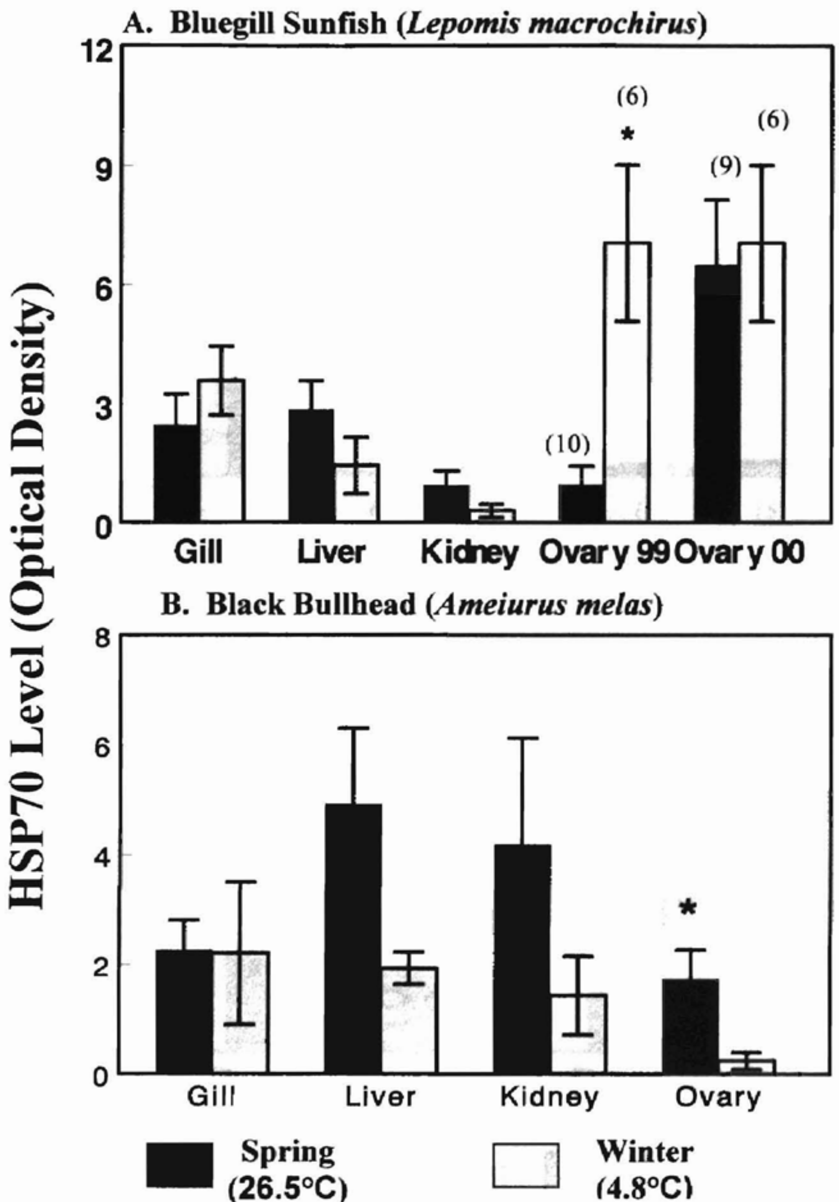

Figure 8. Expression of the 70kDa heat shock protein (HSP70) measured as optical density (mean ± SEM) in gill, liver, kidney and ovary of bluegill sunfish (*Lepomis macrochirus*) (A) and black bullhead (*Ameiurus melas*) (B) collected from Lytle Creek (reference site) during spring (water temperature 26.5 ± 0.95°C; solid bars: n=19 (A), n=11(B)) and winter (water temperature 4.8 ± 0.8°C; shaded bars; n=6(A), n=3(B)) collections. (n) * An asterisk indicates means are significantly different (p<0.05).

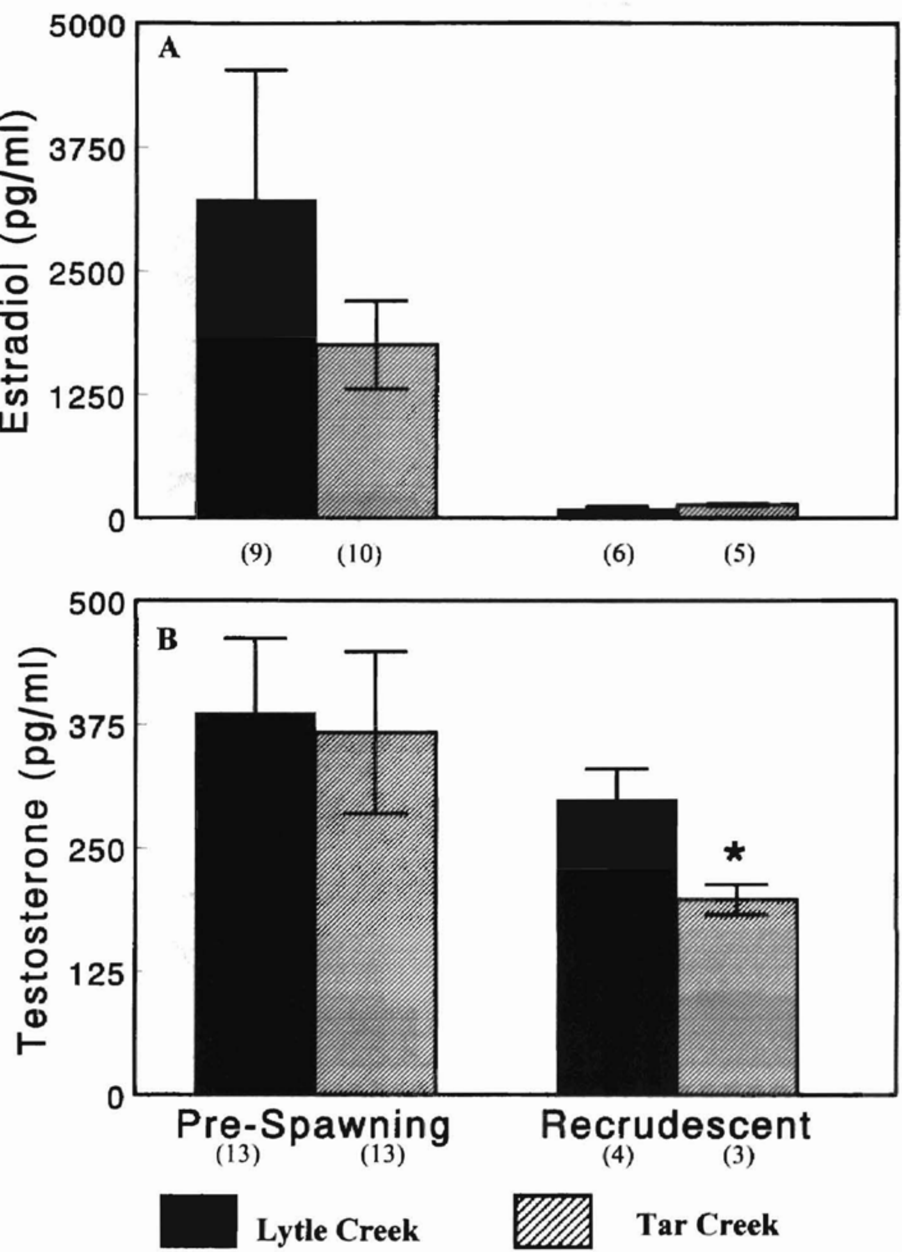

Figure 9. Circulating levels (mean ± SEM) of (A) estradiol (pg/ml) and (B) testosterone (pg/ml) measured by enzyme-linked immunosorbent assay (ELISA) in serum of bluegill sunfish (*Lepomis macrochirus*) collected from Lytle Creek (reference site: solid bars) and Tar Creek (study site: hatched bars) during the pre-spawning (water temperature 26.5 ± 0.95°C) and recrudescent (water temperature 4.8 ± 0.8°C) collections. (n) * An asterisk indicates Tar Creek samples are significantly different from Lytle Creek ($p<0.05$).

Figure 10. Circulating levels (mean ± SEM) of (A) estradiol (ng/ml) and (B) testosterone (ng/ml) measured by enzyme-linked immunosorbent assay (ELISA) in serum of black bullhead (*Ameiurus melas*) collected from Lytle Creek (reference site: solid bars) and Tar Creek (study site: hatched bars) during the pre-spawning (water temperature 26.5 ± 0.95°C) and recrudescent (water temperature 4.8 ± 0.8°C) collections. (n) * An asterisk indicates Tar Creek samples are significantly different from Lytle Creek (p<0.05).

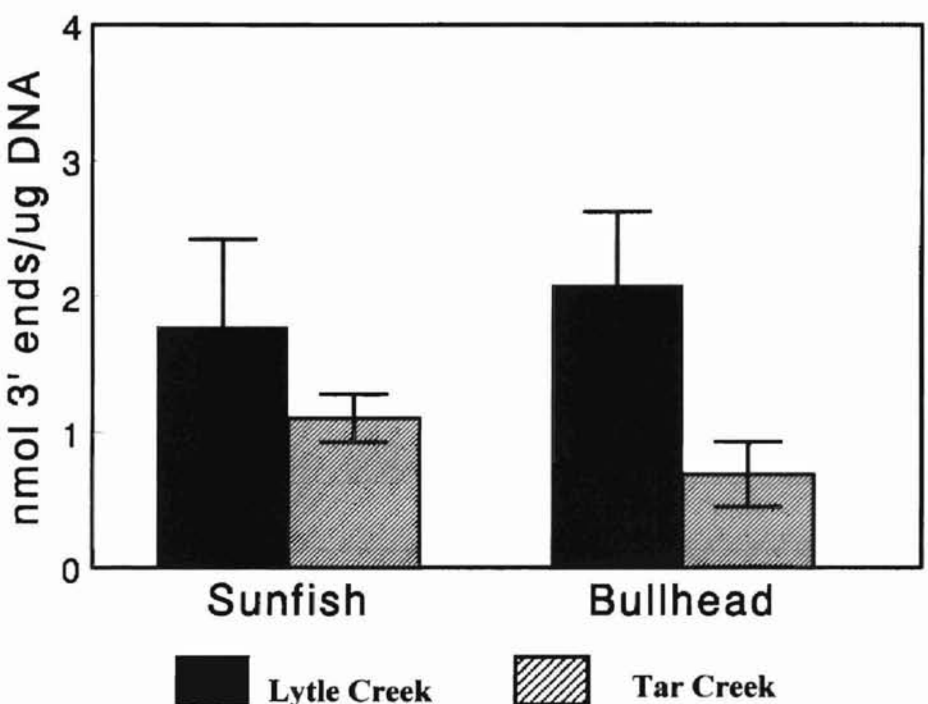

Figure 11. Quantitative estimation of low-molecular weight (<15kb) apoptotic DNA fragmentations in preovulatory bluegill sunfish (*Lepomis macrochirus*) and black bullhead (*Ameiurus melas*) collected from Lytle Creek (reference site: solid bars) and Tar Creek (study site: hatched bars) (mean ± SEM, n=5-6).

were pooled because the means were not significantly different for all organismal responses except bluegill sunfish GSI. GSI was significantly elevated in sunfish inhabiting Tar Creek spring 1999 (p=0.01; n=4-10; Table 3A). No significant differences in condition factor or HSI of bluegill sunfish were determined between sites (Table 3A). The condition factors of black bullhead from Tar Creek during both collection seasons were significantly lower in comparison to Lytle Creek (Spring, p=0.001, n=11-13; Winter, p=0.009, n=2-3; Table 3B). The GSI and HSI were also lower in black bullhead collected from Tar Creek in comparison to Lytle Creek in spring (pre-spawning) collections (p=0.04 and p=0.02, respectively, n=11-13; Table 3B).

A. Bluegill Sunfish *(Lepomis macrochirus)*

Lytle Creek			Tar Creek	
Organismal Responses	**Spring (19)**	**Winter (6)**	**Spring (14)**	**Winter (6)**
Condition Factor= (body wt/length^3) x100,000	1.84 ± 0.12	1.53 ± 0.04	1.90 ± 0.08	1.30 ± 0.18
GSI=gonad wt/(body wt - gonad wt) x 100	4.34 ± 0.73 (1999)(10) 6.36 ± 1.192 (2000)(9)	1.14 ± 0.12	* 8.21 ± 0.96 (1999)(4) 2.78 ± 0.85 (2000)(10)	1.64 ± 0.60
HSI=liver wt/(body wt - liver wt) x 100	1.02 ± 0.09	0.92 ± 0.10	0.86 ± 0.09	1.52 ± 0.47

B. Black Bullhead (*Ameiurus melas*)

Lytle Creek			Tar Creek	
Organismal Responses	**Spring (11)**	**Winter (3)**	**Spring (13)**	**Winter (2)**
Condition Factor= (body wt/length^3) ±100,000	1.39 ± 0.04	1.35 ± 0.03	*1.17 ± 0.04	*1.11 ± 0.02
GSI=gonad wt/(body wt – gonad wt)x 100	1.55 ± 0.49	0.69 ± 0.08	*0.38 ± 0.03	0.77 ± 0.002
HSI=liver wt/(body wt – liver wt)x 100	1.45 ± 0.09	1.83 ± 0.15	*1.17 ± 0.06	1.42 ± 0.20

Table 3. Organismal responses (condition factor, gonadosomatic index, and hepatosomatic index (mean ± SEM)) of bluegill sunfish (*Lepomis macrochirus*) (A) and black bullhead (*Ameiurus melas*) (B) inhabiting Lytle Creek (reference site) or Tar Creek (study site). (n) * An asterisk indicates Tar Creek samples are significantly different from Lytle Creek (p<0.05).

Discussion

Tissue specific expression of HSP70

Fish are susceptible to metal contamination through both aqueous and dietary exposures (Farag et al. 1999; Williams et al. 1996), and physiological impairment in fish inhabiting metal contaminated streams has been reported (Hontela et al. 1995; Farag et al. 1995, 1999). Metals interfere with protein metabolism via disruption of ion balance, displacing essential metal ions of molecules, and interaction with sulfhydryl groups of proteins (McDonald and Wood 1993; Aït-Aïssa et al. 2000). These types of interactions alter the biologically active conformations of proteins and ultimately result in protein degradation (Aït-Aïssa et al. 2000; De Smet and Blust 2001). Metal perturbations of proper protein assembly in cell cultures and whole organism studies have been reported to induce expression of HSP70 in attempt to repair damaged protein structures and prohibit cytotoxicity (Ryan and Hightower 1994; Aït-Aïssa et al. 2000).

HSP70 expression was elevated in only two out of six comparisons of kidney tissue collected from fish chronically exposed to metals. Due to limited sample sizes and variability in the HSP70 response more comparisons were not statistically significant. However, the 2-4-fold induction of HSP70 expression found in all kidney tissues collected from fish inhabiting Tar Creek, with the exception of winter collections of sunfish from Tar Creek having a 21-fold induction, may have biological relevance. Whether or not this level of induction represents proteotoxicity or is within the adaptive response range of these species is unknown. However, these results indicate exposure and a physiological response of the organisms to a quality of Tar Creek that is different

from Lytle Creek. It can be concluded that this physiological response of HSP70 induction may be a threshold response, or early sensitive indicator of toxicity.

Proteotoxicity in the kidney as a result of metal exposure and subsequent elevation of HSP70 would be expected because the kidney is a primary target organ of metal accumulation in both wild fish populations and in laboratory exposures (Farag et al. 1995; Andres et al. 2000; De Smet and Blust 2001). Hontela (1998) reported disruption of interrenal tissue of yellow perch (*Perca flavescens*) occurring as a result of chronic metal exposure, thus interfering with the hypothalamo-pituitary-interrenal axis in fishes. Ryan and Hightower (1994) found concentration-dependent increases of HSP70 in winter flounder (*Pleuronectes americanus*) kidney cell cultures in response to sublethal metal exposure levels prior to cytotoxicity. In mammalian research HSP70 expression is induced in kidney tissue in response to exposure to concentrations of Cd and Hg that cause acute renal cell injury, indicating the role of HSP70 in the cellular defense response to metals (Goering et al. 1993).

In my study I observed differences between species in the level of hepatic HSP70 expression determined in fish chronically exposed to metals. The liver is also a target organ of toxicity for many contaminants, including metals (De Smet and Blust 2001). For example, Weber and Janz (2001) reported elevations in HSP70 expression in liver tissue of juvenile channel catfish (*Ictalurus punctatus*) in response to PAH exposure, and HSP70 was quickly induced in rat liver tissue after exposure to Cd concentrations that did not cause hepatoxicity (Goering et al. 1993). Using liver cell cultures from winter flounder, Ryan and Hightower (1994) reported concentration-dependent increases of HSP70 in response to sublethal Cd exposure levels corresponding with increases in

hepatocellular cytotoxicity. Williams et al. (1996) reported variable levels of HSP70 induction in liver tissue of adult and juvenile rainbow trout exposed to metals (Cd, Cu, Pb, Zn) in both water and food, with a trend for decreased levels of expression.

Metals accumulate differentially in tissues, thereby complicating the tissue specificity of HSP70 expression in response to metal mixture exposures. Nonessential metals, such as cadmium, accumulate in the kidney over long-term exposure because there are no active mechanisms established for the physiological regulation of Cd accumulation, use, and elimination, as demonstrated by McGeer et al. (2000) when chronically exposing rainbow trout (*Oncorhynchus mykiss*) to sublethal levels of Cu, Cd, and Zn. Similarly, Cd accumulated primarily in kidney tissue of carp (*Cyprinus carpio*) in laboratory exposures (De Smet and Blust 2001). The highest zinc concentrations were found in the gill tissue of caged rainbow trout in a metal contaminated river of Italy, while Cd levels were highest in the kidney tissue (Camusso et al. 1995). Lead accumulated most rapidly in the gill tissue of the same fish and subsequently redistributed to the other tissues analyzed throughout the exposure period (Camusso et al. 1995). Zinc and Cd concentrations were highest in the kidney of chub collected along a polymetallic pollution gradient of Lot River, France (Andres et al. 2000).

Concomitantly with accumulation differences metals have differing modes of toxicity and cellular interactions (Reid and McDonald 1988, 1991; Glynn et al. 1994). For example, Zn, Cd, and Pb all induce expression of HSP70 in the slug (*Deroceras reticulatum*), however Cd and Pb induction of HSP70 reached a plateau at higher concentrations of the metals while Zn HSP70 induction increased in correlation with higher exposure concentrations (Köhler et al. 1996). Elevated levels of metals have been

demonstrated to suppress HSP70 expression (Eckwert et al. 1997, Pyza et al. 1997) in comparison to sublethal concentrations in both field and laboratory studies of invertebrates. Duffy et al. (1999) reported decreased expression of HSP70 in subsistence fish with increased water mercury concentrations of the Yukon-Kuskokwim delta region of Alaska, an area polluted by mining operations. Similarly, in mammals, Goering et al. (2000) reported a decrease in the cytoprotective capabilities of heat shock proteins at elevated levels of metals.

Andres et al. (2000) reported that liver concentrations of Cd and Zn did not reflect ambient metal levels of four fish species collected from a polymetallic gradient in the Lot River in France. This result could be associated with large levels of constitutive metallothioneins (MT) found in liver tissues of the common carp (*Cyprinus carpio*) exposed to Cd (De Smet et al. 2001). Metallothioneins are low molecular mass, cysteine-rich, metal-binding polypeptides that are expressed constitutively in tissues for the sequestration of essential metals to maintain cellular homeostasis (Olsson et al. 1987). Metallothionein-metal complexes could accelerate the detoxification of nonessential metals and assist in diminishing proteotoxic interactions of metals to prohibit protein structure disruption and subsequent induction of HSP70. De Smet et al. (2001) also found lower levels of constitutive MT expressed in kidney and gill tissues of the common carp, thus making them susceptible to metal toxicity before MT can be produced to sequester the metals present. These findings support the differences in HSP70 expression determined in kidney tissue, but do not provide explanation for the lack of HSP70 expression in gill tissue in fish chronically exposed to metals.

HSP70 was reported to be elevated in gills of juvenile rainbow trout exposed to metals (Cd, Cu, Pb, Zn) in both water and food (Williams et al. 1996). However, no seasonal or species-specific differences in expression of HSP70 were determined in gill tissue collected from fish chronically exposed to metals in my study. These results are surprising because gills play a significant role in metal uptake directly from the water column. Gill tissue serves as the primary gas and ion exchange site in aquatic species requiring highly evolved cellular mechanisms for the maintenance of membrane structural integrity while being exposed to rapidly changing environmental conditions (pH, temperature, gases, ions) (McDonald and Wood 1993). Cellular mechanisms for maintaining gill membrane integrity include elevated levels of constitutive HSP70, high rates of cellular mitosis and renewal, and elevated mucus production to keep toxic ions removed from gill cells (McDonald and Wood 1993; Schmidt et al. 1998). In agreement with my findings Pedersen et al. (1997) found that HSP70 levels did not increase in gill tissues of shore crabs (*Carcinus maenas*) collected along a trace metal contaminant gradient. Halibut collected from the Hg contaminated Cook Inlet region of Alaska did not have elevated levels of gill HSP70 expression above constitutive levels (Scofield et al. 1999).

The lack HSP70 induction observed in my study could be a result of increased metal tolerance of the fish chronically exposed to metals in Tar Creek. The protective mechanisms of gill tissue contribute to branchial acclimation to chronic sublethal metal exposure in freshwater fish through three processes: (1) increase in mucus production as a physical barrier metal entry, (2) an increase in the storage and detoxification of metal once it has entered the gill tissue through sequestration of metal ions by metallothioneins,

and (3) an increase in resistance of metal-sensitive branchial processes to metal poisoning through alterations in ion regulation (McDonald and Wood 1993).

Reproductive tissues, such as ovarian follicular cells, are highly susceptible to contaminant-induced proteotoxicity. Of specific interest in my study is the effect of chronic metal exposure on ovarian HSP70 expression. Ovarian tissue is particularly susceptible to metal toxicity due to a lack of MT expression and induction (den Besten et al. 1990). If MT is not available to sequester free divalent cations (Cd, Zn), those cations are then available to interfere with normal cellular processes. In my study there were no consistent differences in expression of ovarian follicular cell expression of HSP70 in bluegill sunfish chronically exposed to metals. In the more tolerant species, black bullhead, expression of ovarian follicular cell HSP70 was similar in females from both sites. Similarly, no effects in ovarian HSP70 expression were determined in adult channel catfish exposed to PAHs (Weber and Janz 2001). HSP70 expression was significantly induced in ovarian follicular cells of white sucker (*Catostomus commersoni*) chronically exposed to a mixture of contaminants found in pulpmill effluent (Janz et al. 1997, 2001). In contrast, juvenile channel catfish (*Ictalurus punctatus*) exposed to PAHs were reported to have suppressed expression of HSP70 in ovarian tissue (Weber and Janz 2001), which was negatively correlated with elevated ovarian follicular cell apoptosis in the same fish.

The tissue-specific differences in HSP70 expression in my study are supported by similar findings by Dietz and Somero (1993), who reported significant differences in HSP70 expression between tissues (brain, liver, gill) within individual species and between four marine teleosts tested in response to hyperthermia. Similarly, brown trout

(*Salmo trutta*) showed tissue-specific differences in expression of HSP70 in gill and skin tissues during recovery from exposure to rapid temperature elevations (Schmidt et al. 1998). Fader et al. (1994) reported expression of HSP70 in response to seasonal temperature fluctuations to be species-specific, indicating that each species has constitutive levels in accordance with their ecological and physiological backgrounds.

Although not statistically significant, seasonal variation in HSP70 expression was found in my study. In determining seasonal variation in HSP70 expression in four stream fish species (brown trout, rockbass, yellow bullhead, and fathead minnows) Fader et al. (1994) found HSP70 expression to be highest during spring collections of eurythermal teleost species, but that the level of HSP70 induction was related to constitutive HSP70 levels in individual species. The seasonal differences in the HSP70 level detected in centipedes collected from the same area during different times of the year by Pyza et al. (1997) coincides with the findings Fader et al. (1994) and the results my research. Seasonal differences in HSP70 expression in poikilothermic organisms indicate that different levels are significant for adaptation to periodically changing environments. The seasonal data collected in my research contributes to our knowledge of the physiological and ecological profiles of the organisms studied to accurately assess contaminant exposure and effects.

Steroidogenesis

In my study testosterone was significantly reduced in bluegill sunfish from Tar Creek during the recrudescent stage of ovarian development. Although not statistically significant estradiol was 20-fold lower and testosterone 4-fold lower in black bullhead collected from Tar Creek. Alterations in the ratio of circulating steroid hormones (17β-

estradiol:testosterone) have been reported to be an indication of stress at the organismal level. Reproductive impairment can result when homeostatic ratios of circulating steroid hormones are not present in the organism. White sucker chronically exposed to pulp mill effluent were reported to have decreased serum testosterone and increased serum 17β-estradiol concentrations (Janz et al. 1997) and this was further associated with elevated ovarian follicular cell apoptosis and elevated HSP70 expression. Protogynous teleost (*Monopterus albus*) exposed to elevated Cd concentrations experienced decreased serum levels of testosterone and 17β-estradiol, along with decreased GSIs (Singh 1989). Thomas (1988) reported reduced GSIs, and reductions in circulating 17β-estradiol levels in Atlantic croaker chronically exposed to sublethal levels of Pb.

Alterations in circulating hormones have been reported to occur at all levels of the hypothalamo-pituitary-gonadal axis (Thomas 1989; McMaster et al. 1996b). For example, Thomas (1989) reported accelerated ovarian growth (GSI) and elevated plasma 17β-estradiol as a result of Cd stimulated release of gonadotropins from the pituitary gland of Atlantic croaker (*Micropogonias undulates*). McMaster et al. (1996b) reported perturbations in circulating hormones of fish exposed to industrial wastes as a result of reductions in the steroid biosynthetic capacity of the ovarian follicular cells. Increased HSP70 expression is associated with inhibition of hormone-sensitive steroidogenesis in rat luteal cells (Khanna et al. 1994), however in my study elevated ovarian follicular cell HSP70 expression cannot be associated with suppressed hormone concentrations in either species studied. Reduced levels of 17β-estradiol and testosterone circulating in the serum of black bullhead during the pre-spawning collection could be associated with the significantly reduced GSI, however this is not associated with elevated ovarian follicular

cell apoptosis or HSP70 expression. When measuring circulating hormones, it is necessary to consider the results along with an entire suite of physiological and organismal endpoints because of the integration of multiple levels of endocrine organization in the synthesis of hormones.

Apoptosis

In my study there was no difference in either species between sites in levels of ovarian follicular cell apoptosis. Apoptosis is an active mode of cell death that is induced by a variety of physiological and anthropogenic stimuli. Measuring apoptosis is useful in that it is a sensitive and early indicator of acute and chronic contaminant stress, loss of cellular function and structure, and overall organismal health (reviewed by Sweet et al. 1999). Janz et al. (1997, 2001) reported elevated ovarian follicular apoptosis in pre-spawning white sucker (*Castostomus commersoni*) chronically exposed to pulp mill effluent. Ovarian follicular apoptosis was elevated in juvenile channel catfish (*Ictalurus punctatus*) exposed to PAHs, however a similar response was not induced in adult channel catfish (Weber and Janz 2001). Metals such as Cd have been reported to induce apoptosis (Fujimaki et al. 2000; Kim et al. 2000) through production of reactive oxygen species (ROS), which are potent inducers of proteotoxicity and apoptosis (Risso-de Faverney et al. 2001). Although not statistically significant, the results indicate apoptosis could be lower in fish from Tar Creek. Zinc has been reported to protect some cell types from Cd-induced apoptosis (Szuster-Ciesielska et al. 2000) through inhibition of Cd accumulation in cells, as well as inhibition of Cd-induced production of ROS. Zinc is involved in the maintenance of cellular function and structural integrity (Chai et al. 1999), protecting proteins and nucleic acids from oxidation and degradation caused by

reactive species and free radicals produced by nonessential molecules (i.e. metals). This cytoprotective role of Zn in turn functions to control and suppress apoptotic cell death (Chai et al. 1999).

Recently, low levels of Cd (1, 5, 10 μg/L) have been reported to play a role in increased egg production in Japanese medaka (*Oryzias latipes*) (Peterson et al. SETAC 21st Annual Meeting Abstract). The female fish inhabiting Tar Creek are exposed to elevated levels of Cd in comparison to Lytle Creek, however these concentrations are within acceptable ranges of National Recommended Water Quality Criteria established for chronic Cd exposures to aquatic organisms (Cd 2.2μg/L) (USEPA 1998) and within the ranges used in the above medaka research.

Organismal responses

In my study condition factors were lower in black bullhead inhabiting Tar Creek in comparison to Lytle Creek during both seasons, however GSI and HSI were lower in the pre-spawning collections only. There were no effects of metal exposure on condition factor or HSI in bluegill sunfish. GSI was elevated in bluegill sunfish from Tar Creek during spring 1999 collections, but not different in the subsequent collection. Gross measurements of overall fish health (condition factor, GSI, HSI) are often calculated as an indication of physiological stress at the organismal level. For example, Farag et al. (1995) reported an overall trend for reductions in condition factors of brown trout chronically exposed to metal mixtures found in the Clark Fork River, Montana. In contrast, no differences in HSIs or condition factors were reported in adult yellow perch exposed to a mixture of metals and organic contaminants in the St. Lawrence River (Hontela et al. 1995), however, GSIs were significantly lower in female perch collected

from the contaminated stream. Elevated liver Cd levels in winter flounder (*Pleuronectes americanus*) were associated with reduced GSIs and HSIs (Pereira et al. 1993). Similarly, the GSI of female freshwater catfish (*Clarias batrachus*) were significantly reduced when exposed to sublethal concentrations of Cd (Baile and Kadu 1992). In contrast, elevated Cd was reported to enhance GSIs and increase plasma estradiol circulation in Atlantic croaker (*Micropogonias undulates*) (Thomas 1989), while chronic exposure to sublethal doses of Pb significantly reduced GSIs in female Atlantic croaker (Thomas 1988). As evidenced by the differential responses of organismal indices to metal exposure reported in this and other studies, further work is needed to understand potential relationships between cellular and organismal responses in fish exposed to metals.

Metal levels

Although lower than water concentrations reported previously (U.S. EPA 1994), I determined Cd, Pb, and Zn concentrations of Tar Creek to be significantly higher than metal concentrations in Lytle Creek. Cd and Pb concentrations determined in the dissolved portions of water samples I collected were within the acceptable levels of chronic exposure to aquatic organisms (U.S. EPA 1998), however Zn concentrations in Tar Creek samples far exceed the acceptable level of 120 μg/L proposed by U.S. EPA (1998). The explanation for the lower values found in my study could be related to site differences. Earlier reported values were collected from a site directly upstream from an acid mine drainage during the mid 1980's and further downstream than my sample collections and may have had greater exposure to tailings piles leachate and possible interference of acid mine waste. The site remediation projects (ongoing since 1984)

could be working to decrease the leachate entering the surface water of streams. Hysteresis, a process of natural remediation whereby metals are sequestered over time into the sediment causing a reduction in the bioavailability of the divalent metals, is another possible explanation for the lower concentrations of metals determined in my study.

The bioavailability of the metals in water samples was confirmed by elevated levels of Cd, Pb, and Zn in liver samples of both species collected from Tar Creek regardless of collection season. These data indicate that bluegill sunfish are accumulating metals to a greater degree than the black bullhead species. Depending upon habitat and primary diet, metals accumulate differentially in different species (Norey et al. 1990a). Because sunfish are a more pelagic species than black bullhead, a bottom feeder, it is possible that sunfish are being exposed to the more bioavailable divalent form of the metals dissolved in the water column. Although the fish were accumulating metals in liver tissue, the significantly elevated physicochemical parameters (pH, hardness, alkalinity, conductivity) found at Tar Creek in comparison to Lytle Creek could have ameliorated the toxic effects of metal exposure to fish at the Tar Creek site.

Conclusions

The results of my study should be applied carefully because the study design did not have true replication. Proper replication would have required sampling of both fish species from multiple reference and study (metal contaminated) sites, as well as, samples from multiple sites along each of the two streams. It should be noted, however, that fish collections spanned a 300m stretch of each. The reference and study site are potentially ecologically dissimilar as indicated by the physicochemical parameter differences

measured in each stream. Also, diet of the fish from each site was not analyzed and I cannot rule out the potential for false interpretation of data due to diet dissimilarity between sites in individual species.

The ecological dissimilarity between the streams creates a challenge when assessing the toxicity of metals in a natural setting. However, the goal of the research to gather baseline physiological information for the individual species studied was achieved. My research further confirms the limitations of using HSP70 expression as indicators of contaminant toxicity in a wild population due to the great variability in responses between tissues, seasons, and species. In addition, many biotic and abiotic factors influence the constitutive and induced expression of HSP70, ranging from temperature changes, dietary stress, and reproductive maturity to contaminant exposure. The results of my study are applicable when addressing seasonal differences in each species collected from the reference stream (Lytle Creek), as well as interpreting results based upon species related differences (reproductive strategy, ecology differences).

In summary, HSP70 expression was 2-4 fold higher in the kidney (a major target organ of nonessential metals such as Cd and Pb) of both fish species chronically exposed to metals during both seasons. In contrast, no consistent differences were determined in HSP70 expression in gill, liver, and ovary between metal exposed and reference fish. Distinct differences in HSP70 expression were observed within various tissues of a single species, between species, and from season to season. There was no evidence of ovarian toxicity due to metal exposure based on steroidogenesis, GSI, or ovarian follicular cell apoptosis. By determining differential expression of HSP70 between specific tissues and between species my research will supplement the knowledge of HSP70 expression at the

cellular/molecular and whole organism levels of biological organization, as well provide insight into the relationship between metal exposure and reproductive impairment in wild fish populations.

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Appendix A

A. Bluegill Sunfish (*Lepomis macrochirus*)

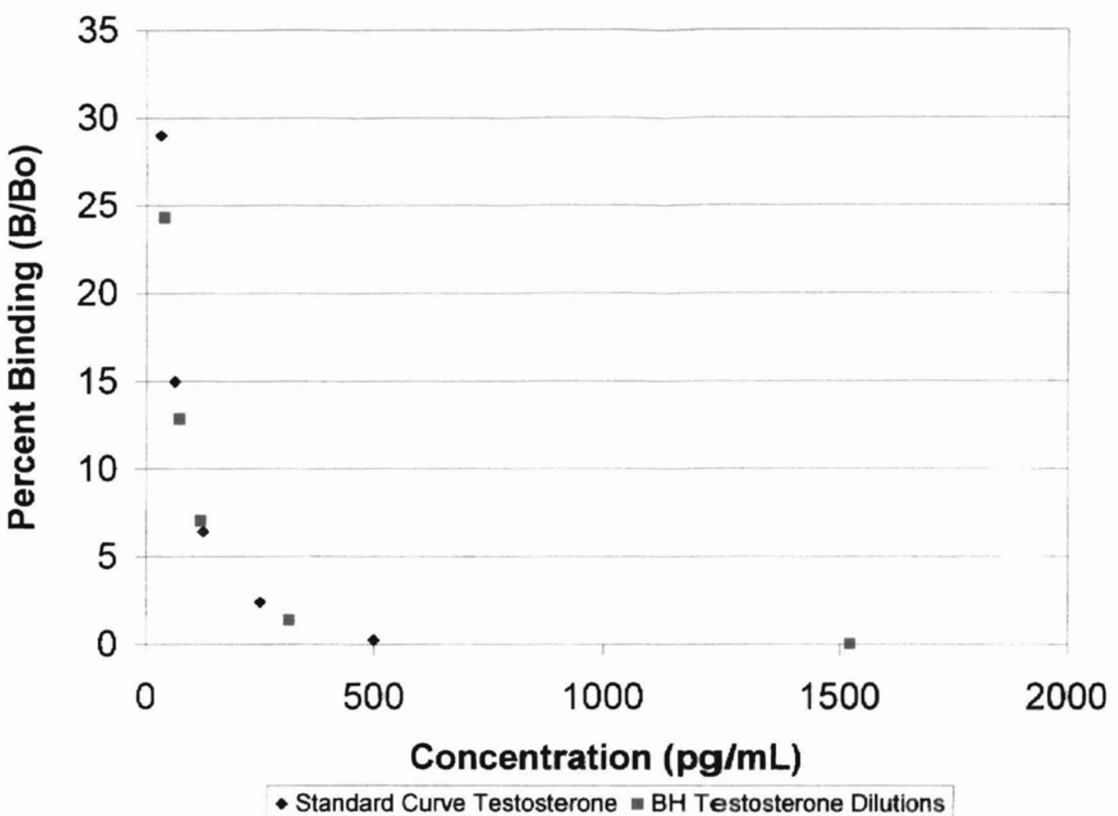
	Lytle Creek			**Tar Creek**		
	Spring 1999 (10)	Winter 2000 (6)	Spring 2000 (9)	Spring 1999 (4)	Winter 2000 (6)	Spring 2000 (10)
Length mm	129.3 ± 4.3	99.7 ± 2.1	92.2 ± 2.3	131.8 ± 2.6	105.3 ± 6.9	104.0 ± 4.9
Weight g	49.0 ± 11.4	15.3 ± 1.0	12.9 ± 1.1	43.9 ± 2.1	17.9 ± 5.2	22.3 ± 3.2

B. Black Bullhead (*Ameiurus melas*)

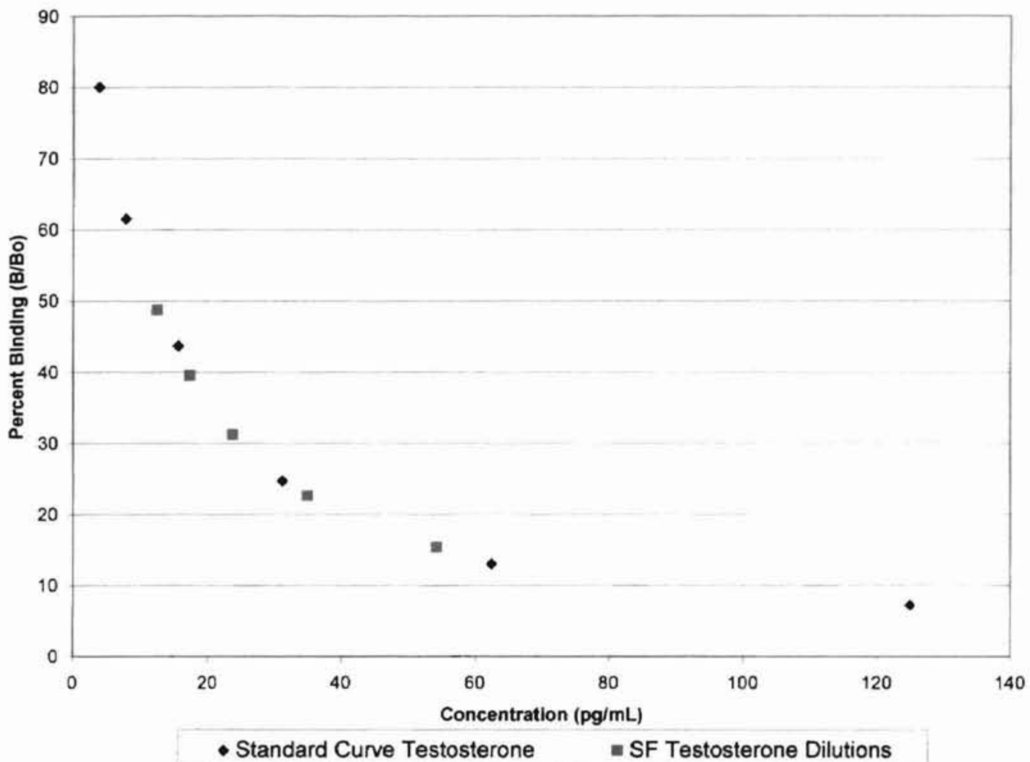
	Lytle Creek			**Tar Creek**		
	Spring 1999 (6)	Winter 2000 (3)	Spring 2000 (5)	Spring 1999 (4)	Winter 2000 (2)	Spring 2000 (9)
Length mm	250.5 ± 10.2	274.3 ± 10.2	277.2 ± 10.2	194.3 ± 14.3	192.5 ± .5	157.9 ± 12.8
Weight g	217.3 ± 23.6	281.6 ± 37.7	311.9 ± 49.7	92.9 ± 19.7	78.9 ± 1.8	55.1 ± 14.1

Length (mm) and weight (g) measurements (mean ± SEM) recorded in the field as bluegill sunfish (*Lepomis macrochirus*) (A) and black bullhead (*Ameiurus melas*) (B) were collected from Lytle Creek and Tar Creek, Oklahoma, during the Spring 1999 & 2000 (water temperature 26.5 ± 0.95°C) and Winter 2000 (water temperature 4.8 ± 0.8°C) (n).

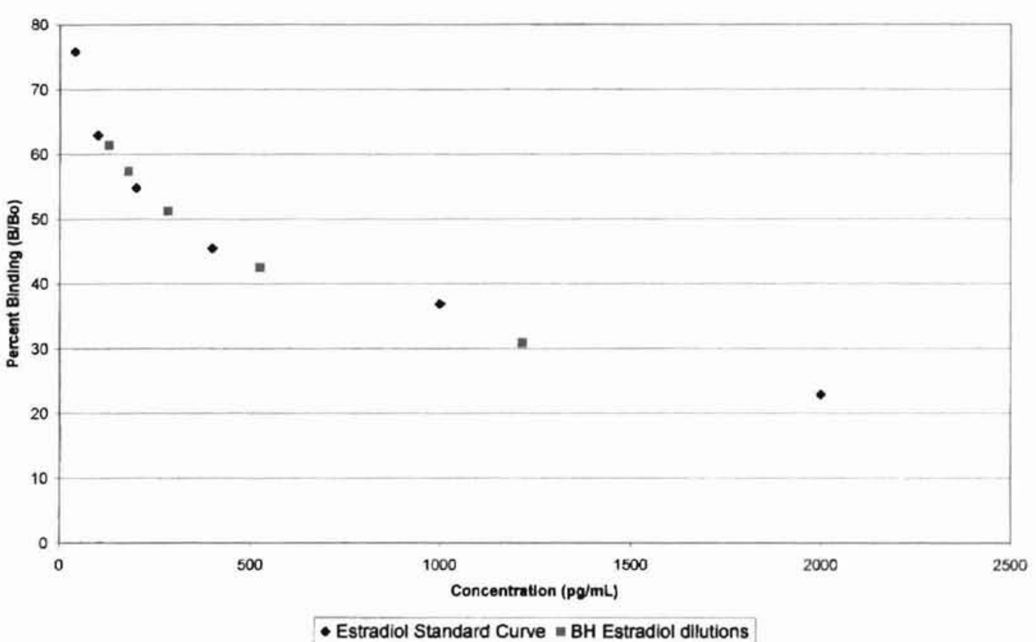
Appendix B



ELISA validation. Serial dilutions of black bullhead (*Ameiurus melas*) serum were assayed by ELISA and compared to testosterone standard curve dilutions. Parallelism was observed.



ELISA validation. Serial dilutions of bluegill sunfish (*Lepomis macrochirus*) serum were assayed by ELISA and compared to testosterone standard curve dilutions. Parallelism was observed.



ELISA validation. Serial dilutions of black bullhead (*Ameiurus melas*) serum were assayed by ELISA and compared to estradiol standard curve dilutions. Parallelism was observed.

Vita

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