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USE OF THE COMMON CARP (CYPRINUS CARPIO) TO MONITOR WASTEWATER EFFLUENTS IN SITU FOR THE PRESENCE OF ENDOCRINE MODULATING CHEMICALS

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USE OF COMMON CARP (*CYPRINUS CARPIO*) TO MONITOR WASTEWATER EFFLUENTS *IN SITU* FOR THE PRESENCE OF ENDOCRINE MODULATING CHEMICALS

By

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ABSTRACT

USE OF COMMON CARP (*CYPRINUS CARPIO*) TO MONITOR WASTEWATER EFFLUENTS *IN SITU* FOR THE PRESENCE OF ENDOCRINE MODULATING CHEMICALS

By

Erin Michelle Snyder

Recent reports have indicated that chemical contaminants entering Lake Mead, Nevada, via the Las Vegas Wash are capable of causing estrogenic (estrogen-like) effects in wild common carp and in an estrogen-responsive cell line. Two separate, but nearly identical, studies were performed using caged carp (*Cyprinus carpio*) to test the Las Vegas Wash influent for the presence of chemical contaminants capable of producing estrogenic effects in fish. In both studies, adult male and female carp were exposed separately in cages (30 fish per cage) at four sites in Lake Mead: one site directly in the influent of the Las Vegas Wash (LW) as it enters Lake Mead, one site in Las Vegas Bay (LX) where the influent of the Wash is more dilute, and two reference sites. Endpoints examined included condition factor (K), gonadosomatic index (GSI), plasma concentrations of sex steroids and vitellogenin (VTG), and histology of the hepatopancreas, ovary, and testis. Plasma sex steroids measured were 17β-estradiol (E2), testosterone (T), and 11-ketotestosterone (11-KT).

The first study (Study I) took place from mid-February to late March or early April; exposure duration ranged from 42 to 48 d. A second study (Study II) was conducted in the same fashion but later in the year, with exposures beginning in late March or early April and lasting through mid-May; exposure duration was 38 to 49 d. Study I: Although LW influent might have altered sex steroid profiles in the carp, it could not be determined conclusively that the effects were due to chemical exposure rather than site-to-site temperature variation. Median plasma VTG concentration in male carp caged in LW was elevated 3- to 10-fold above the concentrations measured in male carp caged at all other sites, indicating that the male carp caged in LW possibly had been exposed to an exogenous estrogenic chemical. However, an effect of site temperature on plasma sex steroid concentrations subsequently resulting in VTG induction cannot be ruled out as a potential cause. Study II: No significant VTG induction was observed in male carp. Mild proliferation of Sertoli cells was observed in the testes of male carp caged at LX. The most interesting findings were possible inhibition of testicular growth in male carp caged in LX and LW and decreased plasma 11-KT concentrations in male carp caged at LW. However, water temperature differences among sites might explain, at least in part, the findings of reduced GSI and plasma 11-KT concentrations; therefore, these conclusions are stated tentatively.

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LIST OF ABBREVIATIONS

VTG ..... vitellogenin
ND ..... not detected
NA ..... not applicable
E2 ..... 17β-estradiol
T ..... testosterone
T-gluc ..... testosterone-glucuronide
11-KT-gluc 11-ketotestosterone-glucuronide
11-KT 11-ketotestosterone
WB ..... Water Barge Cove
MC ..... Moon Cove
LX ..... Las Vegas Bay
LW ..... Las Vegas Wash
HF ..... hatchery fish
GSI ..... gonadosomatic index
K ..... Fulton-type condition factor
kg ..... kilograms
g ..... grams
mg ..... milligrams
µg ..... micrograms
ng ..... nanograms
pg ..... picograms
L ..... liters
mL ..... milliliters
µL ..... microliters
HPLC ..... high pressure liquid chromatography
E2:T ..... estradiol to testosterone ratio
E2:11-KT ..... estradiol to 11-ketotestosterone ratio
E:A ..... estrogen to androgen ratio (E2:T or 11-KT)
µS ..... microSiemens
TIU ..... trypsin inhibition units
RIA ..... radioimmunoassay
ELISA ..... enzyme-linked immunosorbent assay
MDL ..... method detection limit
kD ..... kiloDaltons
nm ..... nanometers
mm ..... millimeters
17-P 17α-hydroxyprogesterone
17,20-P 17α,20β-dihydroxyprogesterone
GtH ..... gonadotropin
GnRH ..... gonadotropin-releasing hormone

HPG-axis.....hypothalamic-pituitary-gonad axis
min .....minutes
hr..... .....hours
d..... days
wk .....weeks
NTU .....National Turbidity Units
dph ..... days post-hatch
PGCs.....primordial germ cells
GVBD..... germinal vesicle breakdown
V .....volts
A ..... amps
UV .....ultraviolet
SDS-PAGE ..... sodium dodecyl sulfate-polyacrylamide gel electrophoresis
PVC ..... polyvinyl chloride
PCBs ..... polychlorinated biphenyls
ECL..... enhanced chemiluminescence
TBST..... Tris-buffered saline with Tween-20
TBST-SG .....Tris-buffered saline with Tween-20 and goat serum
NaCl.....sodium chloride
HCl .....hydrochloric acid
NaOH ..... sodium hydroxide
IgG .....gamma globulin
OPD ..... *o*-phenylene diamine
HRP .....horseradish peroxidase
mM.....millimolar
M..... molar
OD..... optical density
B_o ..... maximum binding
m.....slope of the least squares regression line
b..... y-intercept of the regression line
%CV .....percent coefficient of variation
ANOVA ..... analysis of variance
ANCOVA .....analysis of covariance
°C.....degrees in Celsius
NSB .....non-specific binding
SEM..... standard error of the mean
$CaCO_3$ ..... calcium carbonate
DO.....dissolved oxygen
LH .....leutenizing hormone
FSH ..... follicle stimulating hormone
20β-HSD ..... 20β-hydroxysteroid dehydrogenase
ppm ..... parts per million
NTs..... neurotransmitters
NHs ..... neurohormones

W ..... body weight
L ..... standard length
MSU ..... Michigan State University
USGS ..... United States Geological Survey
SNWA ..... Southern Nevada Water Authority
NDOW ..... Nevada Division of Wildlife
NPS ..... National Park Service
BRD ..... Biological Resources Division
US ..... United States
UK ..... United Kingdom
TIE ..... toxicity identification and evaluation

INTRODUCTION

Lake Mead is a large reservoir formed by impoundment of the Colorado River behind the Hoover Dam. The reservoir serves as a source of domestic and agricultural water for more than 22 million users (LaBounty et al., 1997) and, as part of the Lake Mead National Recreational Area, is used for activities such as boating, swimming, and fishing. The Las Vegas Wash enters the Boulder Basin of Lake Mead via the Las Vegas Bay and provides one the greatest inflows of water into Lake Mead, second only to the Colorado River (LaBounty et al., 1997). The flow of the Las Vegas Wash consists entirely of tertiary treated municipal sewage effluent, urban storm water flow, and groundwater seepage from the urbanized Las Vegas Valley (LaBounty et al., 1997). The water entering Lake Mead via the Las Vegas Wash is considerably more dense and exhibits greater conductivity and turbidity than main body Lake Mead water (LaBounty et al., 1997). The difference in density causes the intrusion of the Las Vegas Wash to form an interflow that extends from Las Vegas Bay into the Boulder Basin. At times, the influence of this interflow has been detected on the basis of its conductivity at the Hoover Dam, 16 km away from the confluence of the Las Vegas Wash with Lake Mead.

In 1996, the United States Geological Survey (USGS) reported evidence that suggested endocrine disruption in wild adult male and female common carp (*Cyprinus carpio*) associated with the Las Vegas Wash and Bay (Bevans et al., 1996). This evidence included alterations in plasma sex steroid concentrations in male and female fish relative to those observed in fish at a reference site and induction of a female-specific protein, vitellogenin (VTG), in the blood of male carp in the absence of a concomitant increase in plasma 17β-estradiol. These findings prompted concern for the razorback sucker (*Xyrauchen texanus*), an endangered species of fish that spawns in the Las Vegas Bay (Holden et al., 1999).

The potential causes of the effects observed in the Lake Mead carp are many, but the induction of VTG in male fish without a concomitant increase in plasma E2 indicated that the fish had been exposed to an estrogen-like (estrogenic) chemical in the environment. VTG is an egg yolk precursor synthesized in the liver of fish in response to the estrogenic sex steroid 17β-estradiol (E2). VTG is transported in the blood to the ovary, where it is sequestered into developing oocytes in preparation for spawning (Mommsen et al., 1988). VTG serves no known function in male fish and normally is not detected (or detected only in very low concentrations) in their blood plasma, presumably because E2 levels are not great enough in male fish to induce VTG synthesis (Mommsen et al., 1988; Specker et al., 1994).

However, male fish possess the VTG gene and are capable of producing VTG when exposed to exogenous estrogenic chemicals (Mommsen et al., 1988; Specker et al., 1994). VTG is a useful biomarker of exposure to estrogenic chemicals in male fish for several reasons. VTG induction is a specific response to estrogenic chemicals, and, in male fish, is attributable to exposure to an estrogenic substance (Mommsen et al., 1988). Because male fish do not possess a normal clearance mechanism for VTG (i.e., deposition into oocytes), it can build to considerable and measurable concentrations in their blood plasma (Mommsen et al., 1988). Also, only a small volume of plasma is needed for measurement of VTG, leaving the remainder for measurement of plasma sex steroids or other hormones that might be important in evaluating potential reproductive dysfunction.

Exposure to an estrogenic chemical also might cause alterations in plasma sex steroid levels in fish, although not necessarily through an estrogen receptor-mediated mechanism (MacLatchy et al., 1997; Tremblay et al., 1998). Given that the Las Vegas Wash consists of wastewater, which can be expected to contain a complex mixture of chemical contaminants, the effects on sex steroids and on plasma VTG in Lake Mead carp might have occurred through entirely different mechanisms and/or by exposure to different chemicals. Studies in the United Kingdom (UK) (Harries et al., 1996; Harries et al., 1997; Harries et al., 1999) and in the United States

(US) (Folmar et al., 1996) on fish in municipal sewage effluent or in rivers receiving it have suggested that contaminants in sewage effluent can cause VTG induction and other reproductive effects in fish. Results of the work conducted in the UK indicated that VTG induction observed in fish in sewage effluents and in some rivers might be caused by water soluble contaminants such as the animal steroids E2 and estrone (E1) and/or an oral birth control medication component, ethinylestradiol (EE2) (Routledge et al., 1998).

To investigate the possibility that water soluble contaminants in the Las Vegas Wash might be causing estrogenic effects in Lake Mead fish, researchers from Michigan State University (MSU) adopted a toxicity identification and evaluation (TIE) scheme to accomplish two goals initially: (1) to determine whether extracts from water samples collected from the Las Vegas Wash (LW) and Las Vegas Bay (LX) were capable of producing an estrogenic response *in vitro* in MCF7-luciferase cells, and (2) to identify likely chemical causes for any *in vitro* responses observed. In 1997, 5 L water samples were collected from LW, LX, and two reference sites in Lake Mead (Saddle Island and Callville Bay) and subjected to solid phase extraction (Snyder et al., 1999). The resulting extracts were fractionated into 3 classes based on polarity and each class was screened for estrogenic activity using an *in vitro* bioassay (MCF7-luciferase) (Snyder et al., 2000b). These same fractions were analyzed for the presence of selected estrogenic

chemicals by separation of analytes with high-pressure liquid chromatography (HPLC) and subsequent detection by fluorescence or radioimmunoassay (RIA). Significant estrogenic responses were induced in the bioassay by the extracts from LW and LX and not by extracts from the reference sites. The bioactivity of these extracts was associated with the fraction of greatest polarity. Mass-balance analysis of known estrogenic chemicals identified in the fractions indicated that steroidal estrogens such as E2 and EE2 were likely causes for the observed bioactivity of the LW and LX extracts. Although they have been implicated as potential causative agents for endocrine disruption in fish exposed to sewage effluent in the UK (Purdom et al., 1994; Harries et al., 1997), alkylphenol ethoxylates and their degradation products, the alkylphenols, are not likely to have contributed significantly to the estrogenic bioactivity of these Lake Mead water extracts *in vitro* (Snyder et al., 2000b).

Although steroidal estrogens appeared to be the most potent estrogenic chemicals in extracts from LW and LX when tested *in vitro*, it cannot be assumed that this would hold true *in vivo* as well. Estrogens produce a variety of effects at multiple targets, some of which involve interaction of different tissues; these types of effects cannot be predicted with a single *in vitro* bioassay (Zacharewski, 1997). *In vitro* bioassays do not account for pharmacokinetics, bioaccumulation, or metabolism (Zacharewski, 1997), all

of which can cause substantial differences in the responses of whole animals versus cells in culture. Therefore, the studies reported here were intended to address the following questions: (1) Can effects observed in wild carp by researchers at USGS be reproduced in caged fish? (2) Are steroidal estrogens and other relatively polar contaminants present in the Las Vegas Wash and Las Vegas Bay at concentrations likely to cause reproductive effects observed in fish? For several reasons, these studies complement the ongoing USGS work with wild carp. The wild carp represent fish receiving natural exposure. Wild carp will feed on detritus in the lake sediment and might be exposed to more hydrophobic chemicals present in food or sediment by this route. They also might receive longer-term exposure or exposure during critical periods of development that might result in effects that would not be detected with a short-term exposure conducted with adult fish. However, the caged fish receive a more controlled exposure with a specified duration and under monitored conditions. Wild fish are free to move in and out of the influence of the Las Vegas Wash, whereas caged fish receive a known level of exposure.

Two separate, but nearly identical, studies were conducted using caged fish to monitor the Las Vegas Wash and Las Vegas Bay for the presence of chemical contaminants with the potential to cause alterations in reproductive endpoints in fish, particularly alterations that would indicate estrogenic

effects. In the first field exposure study (mid-February to late March or early April), adult male and female common carp (*Cyprinus carpio*) were exposed separately in cages (30 fish per cage) for 42 to 48 d at four sites in Lake Mead: one site directly in the influent of the Las Vegas Wash as it enters Lake Mead, one site in Las Vegas Bay where the influent of the Wash is more dilute, and two reference sites. Endpoints examined included condition factor (K), gonadosomatic index (GSI), plasma concentrations of sex steroids and vitellogenin (VTG), and histology of the hepatopancreas, ovary, and testis. Plasma sex steroids measured were 17β-estradiol (E2), testosterone (T), and 11-ketotestosterone (11-KT). The second study was nearly identical, but exposures took place for 38 to 49 d starting in late March or early April and lasting to mid-May. Two nearly identical studies were conducted in an attempt to capture a period of active gonadal growth and development in the carp, to account for fluctuations in contaminants in the Las Vegas Wash, and to determine how potential effects on the fish might vary with time of year or period of seasonal sexual development of the fish.

Chapter 1

USE OF THE COMMON CARP (*CYPRINUS CARPIO*) TO EVALUATE POTENTIAL IMPACTS OF XENOBIOTICS IN SEWAGE TREATMENT PLANT EFFLUENT ON FISH REPRODUCTIVE ENDOCRINOLOGY AND PHYSIOLOGY

Estrogenic, or estrogen-like, effects on reproductive endpoints in fish exposed to sewage treatment plant effluent have been reported in the United Kingdom (Purdom et al., 1994; Harries et al., 1996; Harries et al., 1997; Harries et al., 1999) and in the United States (Bevans et al., 1996; Folmar et al., 1996). In the field of environmental toxicology, methods are being used or developed that utilize the common carp as an experimental model for detecting and studying the impacts of environmental contaminants, particularly those with estrogenic activity, on fish reproductive physiology and endocrinology (Sivarajah et al., 1979; Kumar et al., 1988; Gimeno et al., 1996; Gimeno et al., 1997; Gimeno et al., 1998a; Gimeno et al., 1998b; Smeets et al., 1999). Recent reports have suggested that wild common carp (*Cyprinus carpio*) collected from some areas in the United States have been exposed to estrogenic chemicals, and possibly other endocrine disrupting chemicals (EDCs), associated with sewage treatment plant effluents. Evidence for the occurrence of endocrine disruption in carp included altered sex steroid hormone concentrations or ratios in male and female carp and induction of a female-specific protein, vitellogenin (VTG), in the blood plasma of male carp. Reduced serum testosterone (T) concentrations and elevated plasma VTG concentrations were reported in

male carp collected from an effluent channel below a metropolitan sewage treatment plant (Folmar et al., 1996). In a separate study headed by researchers at the United States Geological Survey (USGS), male and female carp were collected from areas of Lake Mead, Nevada, that are influenced by wastewater from the urbanized Las Vegas Valley (Bevans et al., 1996). Male and female carp demonstrated elevated concentrations of plasma VTG and altered concentrations of plasma sex steroid hormones relative to fish caught at reference sites in the lake.

The presence of elevated concentrations of VTG in the blood of male carp indicated that they were exposed to an estrogenic substance. VTG is an egg yolk precursor synthesized in the liver or hepatopancreas of oviparous vertebrates in response to stimulation by estrogen (17β-estradiol, or E2) secreted by the ovary (Mommsen et al., 1988; Specker et al., 1994). VTG is transported in the blood to the ovary, where it is sequestered into developing oocytes and subsequently cleaved to form lipovitellin and phosvitin, the principle components of yolk (Mommsen et al., 1988). VTG has no known function in male fish and normally is not detected, or detected only at very small concentrations, in their blood, presumably because E2 concentrations are too small in male fish to induce vitellogenesis (Mommsen et al., 1988; Specker et al., 1994). However, male fish possess the VTG gene and can produce VTG under stimulation by exogenous estrogen or

estrogen-like chemicals. Because there is no normal mechanism for clearance of VTG (i.e., deposition into oocytes) in male fish, VTG can build up to great concentrations in their blood (Mommsen et al., 1988). VTG production is a specific response to estrogen and, in some fish species, is highly inducible (Harries et al., 1997); therefore, it serves as a useful biomarker of exposure to estrogenic chemicals. Sexually immature juvenile fish of both sexes also produce only very low levels of VTG, making VTG induction in juvenile fish a useful biomarker as well (Mommsen et al., 1988; Specker et al., 1994).

The results of studies conducted on wild common carp in the United States suggest that carp are responsive to estrogenic chemicals in the environment. Therefore, we proposed the use of caged common carp as part of a toxicity identification and evaluation scheme to identify potential causes for estrogenic effects observed in carp exposed to wastewater entering Lake Mead. The sites selected for study were previously reported to be associated with VTG induction in wild common carp (Bevans et al., 1996), and extracts from water samples taken at these sites caused significant responses *in vitro* in an estrogen-responsive cell bioassay (Snyder et al., 2000a). Researchers in the UK explored the possibility of using caged juvenile common carp to test for estrogenic effects of sewage effluents and found that, in parallel studies with male trout, carp were far less sensitive to

the estrogenic effects of sewage effluents and certain estrogenic chemicals (Purdom et al., 1994). However, they cautioned that the lesser sensitivity of carp might be explained by the fact that exposures took place at temperatures likely to be too low for normal vitellogenesis in carp (Purdom et al., 1994). This hypothesis was supported by another study in which VTG synthesis was induced in male carp held under two different temperature regimes. Intraperitoneal injection of E2 induced vitellogenesis in male carp at 18 to 20 °C but not at 9 to10 °C (Hernández et al., 1992). Although carp are less sensitive than rainbow trout, carp are hardier fish and more likely to survive the conditions encountered in wastewater effluents. In addition, the water temperatures at Lake Mead during the seasons of interest are greater than 10 °C and thus probably conducive to VTG synthesis in carp.

As part of the process of developing the common carp as a fish model for *in situ* reproductive toxicity testing of sewage effluents, some basic information on carp biology, reproductive biology, and reproductive endocrinology was compiled and is presented here.

Common carp as a test species in toxicity studies

The following are properties of the common carp that make it a species useful for environmental toxicology studies. (1) Worldwide, the common carp represents approximately 75% of total freshwater fish production in

aquaculture (Billard, 1999b). Because of its importance as a food fish in other countries, particularly in China, there is a great volume of information available on carp aquaculture and reproduction. (2) The common carp is tolerant of a wide range of water quality conditions; this is particularly important when choosing a species for use in monitoring wastewater effluents, which can vary widely in quality. Carp can tolerate low levels of dissolved oxygen (as little as 2 ppm for brief periods of time) that would be lethal to most other species of fish (Panek, 1987). They also can tolerate high turbidity (Panek, 1987). (3) Sexually mature adults can be obtained that are small enough to handle with relative ease but large enough to provide blood and tissue samples of sufficient size for measurement of multiple reproductive endpoints. (4) The common carp is one of the best-characterized warm water fish with regard to its reproductive physiology and endocrinology such that alterations in the normal state can be detected. (5) The carp has a wide geographical range so that it can be used for environmental monitoring studies in many parts of the world. Effects demonstrated in carp can be applied to risk assessments conducted on wild carp worldwide, with some accounting for differences in strain and variations in reproductive strategy in this species in different geographic regions.

The following are disadvantages associated with the use of common carp as a test species. (1) Carp can be less sensitive to some environmental

contaminants than other potentially more economically or aesthetically important species, such as trout (Purdom et al., 1994). (2) In the United States, the carp is considered to be a pest species and not necessarily deserving of protection. Negative reproductive effects observed in carp are not likely to draw much interest from the public. (3) Carp are not strongly sexually dimorphic and are difficult to sex accurately outside the spawning season without using invasive methods. (4) There are few commercial suppliers of common carp in the United States. (5) The cycle of ovarian development (asynchronous ovary- discussed below) and associated changes in reproductive endocrinology in carp are complex and can vary with geographic region and strain of fish; this can complicate the interpretation of chemically-induced alterations in reproductive endpoints.

General biology

The common carp is a teleost (ray-finned bony fish) belonging to the family Cyprinidae (cyprinid fishes). The cyprinid fishes (carps and minnows) also include two other important freshwater fish species commonly used in scientific research, the fathead minnow (*Pimephales promelas*) and the goldfish (*Carassius auratus*). For the purposes of this work, the term "carp" may include the common carp, *Cyprinus carpio*, and the different varieties of this species including the Japanese ornamental koi carp and four European varieties (sometimes termed "table carp") bred for fast growth, meat

production, and sometimes easier scaling (Huner et al., 1984). The European varieties are named for their scale patterns ranging from fully scaled to scaleless; these include the scale carp, mirror (Israeli) carp, line (Zeil) carp, and leather carp (Huner et al., 1984).

The common carp has been introduced in North America and now thrives over a large range, including most of the contiguous United States, and in a variety of aquatic ecosystems (Boschung et al., 1995). Common carp originally were introduced in United States in the late 1880s as sport fish but never became particularly popular with anglers and are now considered to be a pest (Boschung et al., 1995). Complaints against the carp include that it competes with native fishes for resources, destroys aquatic vegetation, and increases water turbidity by foraging in the sediment for food (Billard, 1999b). Carp are raised in a few aquaculture operations in the United States, primarily to supply Asian and Eastern European communities with food fish. Carp are used as food fish and raised in aquaculture in countries outside North America, particularly in Asia, Eastern Europe, and Israel, and hence a large proportion of the available literature on carp culture and reproduction comes from these parts of the world.

Reproductive strategy

Carp are oviparous (eggs are fertilized and hatch outside the body) and iteroparous (i.e., they spawn more than once in a lifetime). The number of spawnings per year varies with geographic region, such that carp may spawn only once a year in some colder regions (Aida, 1988) and two or more times in a year in warmer locations (Boon Swee et al., 1966). In North America, spawning usually begins in spring (Peter, 1981) and may last through early fall (September). In the wild, carp gather in groups of both sexes or with several males per female (polyandrous spawning) to spawn in vegetation found in shallow water (Boon Swee et al., 1966). Males drive the females into the vegetation, where females release their eggs and males release sperm (Boon Swee et al., 1966). The eggs are adhesive and cling to the vegetation where they remain until hatching. Carp do not exhibit any parental care of eggs or offspring.

Sex determination

Carp are subject to genotypic XY sex determination, with the female as the homogametic sex (XX) and the male as the heterogametic sex (XY) (Komen et al., 1992; Gimeno et al., 1996). Carp are phenotypically sexually undifferentiated for the first few weeks post-hatch (Komen et al., 1992). In addition to genetic control, hormones and other factors also influence the

development of reproductive structures and sexual differentiation of somatic tissue in fish (Redding et al., 1993).

The timing of sexual differentiation varies with temperature and with sex. In a genetically all-male population of common carp (resulting from fertilization of XX eggs with sperm from a YY male) held at 25 °C, sexual differentiation began at 50 days post-hatch (dph) (Gimeno et al., 1997). At that time, primordial germ cells (PGCs), which later develop into gametes, began to proliferate (Gimeno et al., 1997). In a separate study, carp from a normal cross were held at 24.5 °C. Sexual differentiation was first observed in these fish at 70 dph in the female gonad and at 110 to 120 dph in the male gonad (Komen et al., 1992).

Primary sexual characters

Primary sexual characters consist of the gonads (testis in the male, ovary in the female) and associated ducts and glands (spermiduct, oviduct). For reviews on teleostean gonad structure and development, see (Dodd et al., 1984; Redding et al., 1993; Norris, 1997a).

Testes

The testes are bilaterally paired structures located in the coelemic cavity. They are whitish or cream-colored and can occupy a significant portion of

the abdominal cavity when fully developed prior to spawning (Gupta, 1975). The organization of the carp testis has been described as a tubular-type arrangement, with each tubule surrounded by cysts (Komen et al., 1992). Cysts consist of close associations between isolated nests of spermatogonia and Sertoli cells (nurse or cyst cells) (Komen et al., 1992). Sertoli cells phagocytize excess cytoplasm (residual body) that is extruded during spermiogenesis (discussed below) and any germ cells that undergo apoptosis (Miles-Richardson et al., 1999). Germ cells in the same cyst are usually all in the same state of maturation (Gupta, 1975; Nagahama, 1983). As spermatogonia proliferate and differentiate during gametogenesis, the spermatids and spermatozoa are found in the lumina of the cysts (Komen et al., 1992; Gimeno et al., 1998a). During spermatogenesis and spermiogenesis, the cysts expand and then rupture to release the sperm (Gupta, 1975) into the lobular lumen, which is continuous with the spermiduct (Nagahama, 1983). Tubules are separated by bands of connective tissue interspersed with capillaries and steroid-secreting Leydig cells (interstitial cells) (Komen et al., 1992; Redding et al., 1993). Gimeno et al. (Gimeno et al., 1998a) and Gupta (Gupta, 1975) described the structure of carp testis as lobular rather than tubular. In the lobular testis, connective tissue septa running inward from the outer stroma divide the testis into lobules (Gupta, 1975). As spermatogenesis progresses, the stroma and interlobular septa stretch thin and become less obvious (Gupta, 1975). The

entire testis is surrounded by stroma, which consists of loose fibrous connective tissue, and a thin membrane, the peritoneum, which rests on the stroma (Gupta, 1975).

Ovaries

In common carp, the ovaries, like the testes, are bilaterally paired structures found in the coelemic cavity (Gupta, 1975). When the fish are ripe for spawning, the ovaries can occupy an even larger proportion of the coelemic cavity than the testes do in the male. As oocytes begin to develop, they are visible through the ovarian epithelium upon gross inspection of the ovary. Oocytes may appear whitish to yellow or yellow tinged with orange. Immediately following the reproductive season and prior to oocyte growth for the next spawning period, the ovaries may appear as flaccid streaks of translucent pink or pale gray tissue with no oocytes evident upon gross inspection of the external ovary. With some practice, the ovary usually can be readily distinguished from a testis even in fish in post-spawning condition. Carp are iteroparous (spawn more than once) and have an asynchronous ovary, with oocytes at various stages of development present simultaneously so that successive spawnings can take place (Gupta, 1975). This is in contrast to semelparous species, like certain salmonids, which spawn only once and exhibit a completely synchronous ovary with oocytes all at the same stage of development. Because the carp has an asynchronous ovary,

a partially spent ovary usually is readily distinguished from an undeveloped testis by the remaining oocytes developing for the next spawning event (Gupta, 1975). Each carp ovary is covered with a thin layer of connective tissue (tunica albuginea) and then a layer of peritoneum (Gupta, 1975). Lamellae consisting of connective tissue, capillaries, and germinal epithelium extend into the interior of the ovary from the tunica albuginea and support the developing ooyctes (Gupta, 1975).

Accessory ducts

Ducts associated with the gonads in common carp are the oviduct in the female and the spermiduct (vas deferens, see below) in the male. Carp ovaries join at the posterior end and then open to the exterior (Gupta, 1975). This is described as the cystovarian condition, in which ova are released into oviducts that are continuous with the covering of the ovary and then expelled from the body rather than being released to the body cavity and then funneled into the oviduct (gymnovarian condition) (Bond, 1996). Carp testes also join posteriorly, and milt is ejected from a common duct (Gupta, 1975).

Development of gonads and accessory ducts

Normal development of the gonads in common carp is described elsewhere (Komen et al., 1992). Sex steroids probably play an important role in

development of the gonads and reproductive tract in sexually immature fish (Redding et al., 1993). In fact, administration of sex steroids (natural or synthetic) to young fish undergoing sexual development can cause partial or full phenotypic sex reversal in carp (Nagy et al., 1981; Komen et al., 1989), as well as in other species. Use of sex steroids for this purpose is common in aquaculture, where selection of a specific sex may be desired if one sex grows more quickly or provides better quality flesh.

Primordial gonads

Club-shaped primordial gonads develop bilaterally on either side of the swim bladder (Komen et al., 1992; Gimeno et al., 1997). The primordial gonads are attached dorsally to the intestinal coelemic epithelium by a band of stromal cells and protrude into the coelemic cavity (Komen et al., 1992; Gimeno et al., 1997). Germ cells (primordial germ cells, or PGCs) begin to proliferate rapidly until they are present along the entire length of the gonad (Komen et al., 1992). The PGCs are undifferentiated and have the potential to develop into either spermatogonia or oogonia (Komen et al., 1992). The primordial gonad grows larger and becomes filled with somatic cells, connective tissue, and capillaries (Komen et al., 1992). At this point, morphological sexual differentiation begins (Komen et al., 1992).

Testes

In males, the primordial gonads develop into a mass of connective tissue, capillaries, germ cells, and cyst cells (Komen et al., 1992; Gimeno et al., 1997). Cyst cells and germ cells exist in close association (as decribed above), and as the germ cells (now primary spermatogonia) begin to proliferate, the cyst cells enclose them (Komen et al., 1992). Following mitotic and meiotic divisions, spermatids and mature spermatozoa are seen within the lumina of the cysts (Komen et al., 1992; Gimeno et al., 1997).

Ovaries

In female carp, the initiation of formation of an enclosed ovarian cavity is the first obvious sign that gonadal differentiation is taking place (Komen et al., 1992). Stromal cells on the distal end of the gonad begin to extend toward the wall of the coelemic cavity until they meet a similar extension of stromal cells extending from the coelemic epithelium to form a closed cavity (Komen et al., 1992). Germ cells migrate to the portion of the ovary opposite the ovarian cavity and begin to undergo oogenesis (Komen et al., 1992).

Accessory ducts

Teleosts, including common carp, develop spermiducts that are derived from the coelemic walls and thus are not homologous to the vasa deferentia, which develop from the wolffian ducts in mammals (Norris, 1997a).

Similarly, in most female teleosts, the oviduct is continuous with the germinal epithelium covering the ovary and is not homologous to the mammalian oviduct, which is derived from the müllerian duct (Norris, 1997a).

Secondary sexual characters

Outside the reproductive season, it can be very difficult to readily distinguish between the sexes by non-invasive methods (Boon Swee et al., 1966). Sexually mature male carp may develop nuptial tubercles, or pearl organs (tiny, whitish or colorless, horn-like projections), on their heads and pectoral fin rays; these tubercles are absent in females (Boon Swee et al.,). When ripe, males can be distinguished by the ejection of sperm (milt) from the urogenital opening when gentle pressure is applied to the abdomen (stripping) (Huner et al., 1984). Females are identified by the reddened and swollen appearance of the urogenital opening (Huner et al., 1984). When observed from above, a ripe female will appear broader across the abdomen than a male or unripe female (Boon Swee et al., 1966). In some populations of carp, the males that are prepared to spawn exhibit more red and yellow coloration, particularly on the ventral aspect of the abdomen, than do females (personal observation). Gonadal steroids are necessary for the development and maintenance of secondary sex characters such as nuptial tubercles, which are androgen-dependent structures (Norris, 1997a).

Sex Steroids

In female carp, and in female teleost fish in general, the most important sex steroids appear to be the estrogen 17β-estradiol (E2) and the androgen testosterone (T) (McMaster et al., 1995). During gonadal recrudescence (seasonal gonadal growth), T serves as a precursor for E2, which in turn induces vitellogenesis (McMaster et al., 1995). In male carp, the predominant sex steroids are the androgens T and 11-ketotestosterone (11-KT). In male teleosts, T and 11-KT are elevated prior to spawning and are thought to regulate spermiogenesis and spermatogenesis, respectively (Barry et al., 1990a). 11-KT appears to be the principal androgen produced by carp testes (Barry et al., 1989; Barry et al., 1990a) and by the testes of male teleosts in general (Kime, 1979). 11-KT also is produced in the teleost ovary (Norris, 1997a) and has been measured in blood plasma of female carp (Bevans et al., 1996; Goodbred et al., 1997). T serves as a precursor for synthesis of 11-KT. T is an aromatizable androgen, i.e., it is a substrate for the enzyme aromatase, which converts T to E2, while 11-KT is a non-aromatizable androgen (Pasmanik et al., 1988). Although estrogens are generally considered to be female hormones and androgens to be male hormones, both androgens and estrogens are present in both sexes. At certain times in the reproductive cycle, androgen levels may exceed estrogen levels in female fish, including carp (Santos et al., 1986), and androgens (T) in pre-spawning females can reach levels greater than those found in male

fish (Santos et al., 1986; Barry et al., 1990b), suggesting a behavioral role for androgens in female fish (Norris, 1997a). The sex steroids in fish are important for development and maintenance of primary and secondary sex characters, recrudescence and gametogenesis, and sexual behavior (Redding et al., 1993; Norris, 1997a; Nakamura et al., 1998).

Hypothalamic-pituitary-gonad axis

Fish, like mammals, have a hypothalamic-pituitary-gonad axis (HPG-axis) which controls the activities of the reproductive system (Figure 1-1). Sensory structures receive external and internal stimuli and cause the brain (including the hypothalamus) to release neurotransmitters and neurohormones. Neurotransmitters (NTs) and neurohormones (NHs) alter the activities of the hypothalamus and pituitary (hypophysis). Hormones from the hypothalamus stimulate or inhibit synthesis and secretion of pituitary hormones, which, in conjunction with other factors, control the activities of the gonads (steroidogenesis, gametogenesis). The gonads synthesize and release sex steroids and non-steroid factors that feed back negatively or positively at the various levels of the HPG-axis. For reviews, see (Norris, 1997a; Van Der Kraak et al., 1998).

With regard to endocrine control of reproduction in fish, the HPG-axis model parallels the standard mammalian-type model. Gonadotropin-releasing

hormone (GnRH) released from the hypothalamus induces gonadotropin (GtH) release from the pituitary. In carp, there are two gonadotropins, GtH-I and GtH-II (Van Der Kraak et al., 1992). The GtHs control gametogenesis and steroidogenesis in the gonads. In the testis, androgens appear to be produced primarily by the Leydig cells (Yaron, 1995) or other somatic tissues of the testis (Nagahama, 1987; Barry et al., 1990a). In the ovary, the granulosa and thecal cell layers that surround a developing oocyte in an ovarian follicle secrete sex steroids (Nagahama, 1987). Sex steroids (e.g., E2, T, 11-KT) and other factors produced in the gonads feed back either positively or negatively at the gonad, pituitary, hypothalamus, and brain (Van Der Kraak et al., 1998).

Steroidogenesis

The pathway of ovarian steroidogenesis begins with cholesterol as a precursor (Figure 1-2). Regulation of steroidogenesis in fish probably occurs at cholesterol side chain cleavage (Kime, 1979), where cholesterol is converted to pregnenolone by cholesterol side chain cleavage enzyme (SCC).

Gonadal recrudescence

In preparation for spawning, the gonads of male and female carp undergo gonadal recrudescence, or seasonal sexual development, characterized by gonadal growth and development. During gonadal recrudescence, VTG

accumulation in the ovarian follicles is responsible for the majority of ovarian growth (Tyler et al., 1996). After spawning, the gonads undergo a resting period (they are regressed), characterized by a reduction in gonad size and activity, until the onset of the next period of recrudescence. The reproductive state of the fish relative to these gonadal recrudescence cycles can be determined by examining the gonadosomatic index (GSI), or the ratio of the mass of the gonad to the mass of the entire carcass or gutted carcass, where GSI = [(gonad mass/body mass)*100] (Tables 1-1 and 1-2).

Carp in North America, Poland, Japan, and other temperate regions typically spawn in spring (Bieniarz et al., 1978; Peter, 1981; Aida, 1988). After a brief interlude, ovarian recrudescence and vitellogenesis initiate in the female carp during the summer when temperatures are still high (Bieniarz et al., 1978; Peter, 1981). During gonadal recrudescence in female carp, plasma GtH concentration increases and remains elevated (Bieniarz et al., 1978), presumably stimulating E2 synthesis, which in turn stimulates the vitellogenesis which is responsible for oocyte growth during this period. During the colder fall and winter months (October through spring), GtH levels decline (Bieniarz et al., 1978) and vitellogenesis and ovarian growth pause. After vitellogenesis is completed, the oocytes may be maintained without degradation for several months if fish are kept at or below 16 °C (Aida, 1988). Ovulation and spawning take place within a few days when the

water temperature increases to approximately 20 °C (Aida, 1988). Carp in temperate regions may undergo a second period of ovarian growth in the spring prior to spawning. Female carp examined in Poland experienced a second increase in plasma GtH in May during ovarian maturation and spawning (Bieniarz et al., 1978). Plasma GtH increases in mature females when males are present, remains elevated during spawning, and decreases after spawning (Peter, 1981). Female carp also experience a periovulatory surge of gonadotropin that appears to be involved in final oocyte maturation and ovulation (Bieniarz et al., 1978).

Adult male carp are in active spermatogenesis year-round, and at least a small volume of milt usually can be obtained from them even during inter-spawning periods if temperatures are not too low for spawning (Shikhshabekov, 1972). However, male carp in temperate regions also undergo a gonadal cycle characterized by increases and decreases in GSI (Shikhshabekov, 1972), although the fluctuations in GSI are not so dramatic as those observed in females. Wild carp in Dagestan, Russia, spawn from the latter half of April through mid-August (Shikhshabekov, 1972). Among these wild male carp, GSI in males increased in September-October to 80% of the yearly maximum, then increased again to the yearly maximum GSI in March-April just prior to spawning (Shikhshabekov, 1972).

Interestingly, in warmer parts of the world such as India, carp will spawn twice or even continuously in a single year (Parameswaran et al., 1972), so geographic region and temperature should be taken into account when determining the reproductive state of carp. Temperature, and thus geographic region, also has an effect on the time to sexual maturity in carp. In Central Europe, male and female carp typically reach maturity in the third and fourth summers, respectively; while in Japan, females mature in the second year and males in the second or first year (Parameswaran et al., 1972). Both sexes mature in the first year in Israel and in the first six months in Malaysia and India (Parameswaran et al., 1972). Carp raised in southern California typically reach maturity in 2 years (J. Young, personal communication). Rearing carp at elevated temperatures accelerates the development of the gonads in both sexes (Gupta, 1975). The importance of temperature on carp reproduction will be discussed further below. It also has been noted that males tend to reach sexual maturity sooner than females, and larger fish generally mature sooner than smaller fish in the same geographic region. Also, wild carp and carp held in ponds in the same geographic region might spawn with different frequencies (Parameswaran et al., 1972).

Gametogenesis and steroidogenesis

Oogenesis and ovarian development

Excellent reviews of teleost oocyte growth and development are available elsewhere (Wallace et al., 1981; Tyler et al., 1996). The stages of ovarian development in carp, including gross morphology of the ovary at the various stages, have been described in detail (Gupta, 1975). Briefly, stage 1 and stage 2 ovaries contain pre-vitellogenic follicles of the primary growth stage or early yolk vesicle (cortical alveolus) stage; oocytes in these stages have a diameter $\leq$ 0.3 mm (Gupta, 1975). Stage 3 ovaries (advanced maturing) contain vitellogenic follicles with oocytes of >0.3 - 0.7 mm in diameter. Larger oocytes in this stage contain yolk globules, which, at the end of this stage, push the yolk vesicles to the periphery of the ooplasm. Finally, the yolk globules fuse to form a continuous yolk mass at the center of the oocyte. Stage 4 (mature) ovaries contain oocytes with diameter ~0.80 - 0.85 mm. These oocytes are filled with yolk and a few vacuoles. The nuclear membrane disintegrates (Gupta, 1975) in a step known as germinal vesicle breakdown (GVBD). GVBD signifies the onset of final oocyte maturation (Nagahama, 1987). In stage 5 ovaries, the oocytes attain their maximum diameter of ~0.9 mm or greater. GVBD is complete so that nuclear contents are no longer visible in the oocytes, and ovulation begins. The weight of the ovaries may represent 20% of total body weight (Gupta, 1975) or greater at the time of ovulation (Table 1-1).

Steroidogenesis in relation to ovarian recrudescence

In carp, as in some other species, the pattern of steroidogenesis changes with gonadal maturation. Stage 1, stage 2, and early stage 3 ovaries contain pre-vitellogenic oocytes. Toward the end of the primary growth phase, the oocytes enter the follicular phase during which the oocytes become invested by layers of steroid-secreting granulosa and thecal cells (Tyler et al., 1996). An ovarian follicle consists of an oocyte and its associated granulosa and thecal cell layers enveloped by a surface epithelium (Tyler et al., 1996). Early stage 3 follicles produce very little T or E2 (Manning et al., 1984). Late stage 3 ovarian follicles primarily produce E2, which stimulates vitellogenesis (Manning et al., 1984). VTG accumulation accounts for the majority of oocyte growth (Tyler et al., 1996). As the ovaries mature to stage 4 or stage 5, there is a steroidogenic shift to production of T by ovarian follicles (Manning et al., 1984). A GtH surge occurs just prior to ovulation and induces final oocyte maturation (Tyler et al., 1996), a step necessary for fertilization to occur. Increased production of T near the end of vitellogenesis might trigger the GtH surge (Van Der Kraak et al., 1998).

Vitellogenesis

VTG is a large phospholipoglycoprotein egg yolk precursor synthesized in the liver of oviparous vertebrates in response to estrogens (Mommsen et al., 1988). VTG is selectively sequestered from the blood into developing oocytes in the ovaries of female fish undergoing gonadal recrudescence (Mommsen et al., 1988). Incorporation of VTG is responsible in large part for the increase in size of developing oocytes (Tyler et al., 1996). Inside the oocyte, the VTG is cleaved to the major yolk constituents lipovitellin and phosvitin (Mommsen et al., 1988). The primary estrogen responsible for stimulation of VTG synthesis is the most potent endogenous (physiological) estrogen, E2 (Mommsen et al., 1988). E2 is synthesized in the gonad in response to GtH. GtHs also may be involved in stimulation of oocyte uptake of VTG and final oocyte maturation (Tyler et al., 1996).

Oocyte maturation

In teleosts, there is a shift from production of androgen to progestogen during the latter part of gamete maturation in both males and females (Abdullah et al., 1994). In females, T and E2 production declines as 17α,20β-dihydroxy-4-pregnen-3-one (17,20-P) production increases. 17,20-P is synthesized from the substrate 17α-hydroxyprogesterone (17-P). This shift in steroid production is concomitant with a GtH surge that occurs near final oocyte maturation (Abdullah et al., 1994). In female carp, substrate

17-P concentration is known to influence the steroidogenic pathway. As substrate 17-P concentration increases, a shift occurs from 7α-hydroxylated metabolites to 17,20-P (Abdullah et al., 1994). Thus, high levels of steroids that are important for gonadal development, sexual behavior, and secondary sex characteristics are needed during gametogenesis until just prior to spawning; when there is a shift to 17,20-P, which is responsible for final oocyte maturation and spermiation (Abdullah et al., 1994) (Figure 1-2).

Spermatogenesis and testicular development

Early in testicular development, the immature testes have an appearance similar to undeveloped ovaries, and histological examination may be required to confirm the sex of the fish (Gupta, 1975). When the testes are fully developed, they are white or cream-colored and occupy a significant proportion of the body cavity (Table 1-2), and milt is readily released when the abdominal wall of the male is gently pressed (Gupta, 1975). Spermatogenesis in male cyprinids is similar to that in other vertebrates (Komen et al., 1992; Redding et al., 1993; Norris, 1997b). A diploid germ cell (spermatogonium, or spermatogonial stem cell) undergoes mitotic proliferation, and some of the spermatogonia differentiate to committed primary spermatocytes. Each spermatocyte undergoes meiosis (spermatogenesis) to become two secondary spermatocytes. Each secondary spermatocyte undergoes a second meitoic division to yield two

haploid spermatids. Spermiogenesis, which occurs prior to spawning, is the transformation of spermatids to mature spermatozoa with characteristic flagella (Redding et al., 1993). The spermatozoa are first located in the cysts of the seminiferous lobules, but eventually collect in the lumen of the testis and are ejected through the spermiducts (spermiation) during spawning. GtH is the major hormone that regulates spermatogenesis; it induces proliferation of the spermatogonia and induces steroidogenesis in the Leydig cells (Redding et al., 1993).

Spermiation

The 11-oxygenated androgens (such as 11-KT) are the predominant steroids during gonadal recrudescence in the males of many species of fish (Abdullah et al., 1994). 11-KT appeared to be the dominant androgen produced by testicular fragments taken from spermiating carp and incubated *in vitro* at 20 °C (Barry et al., 1990a). In male cyprinids, there is a shift from the production of 11-oxygenated androgens to progestogens, including 17,20-P, near the time of spawning, just before or during spermiation (Nagahama, 1987). 20β-hydroxysteroid dehydrogenase (20β-HSD), the enzyme responsible for conversion of 17-P to 17,20-P is localized in the sperm (Barry et al., 1990a). It has been suggested that during the pre-spawning GtH surge, $C_{17\text{-}20}$lyase (Figure 1-2), the rate-limiting enzyme in androgen biosynthesis, becomes saturated with substrate 17-P, which diffuses out of

the somatic cells to the spermatozoa and is metabolized to 17,20-P (Barry et al., 1990a). 17,20-P exerts a negative feedback on androgen production in the somatic cells, resulting in the rapid drop in androgen levels and rapid increase in 17,20-P that occurs during spermiation in carp (Barry et al., 1990a).

Ovulation and spawning

Ovulation

Methods for inducing ovulation in female carp have been described (Santos et al., 1986; Aida, 1988; Yaron, 1995). In one study, water temperature was increased from 16 to 24 °C (heat hypophysation) (Santos et al., 1986). Changes that resulted were described as follows. Most females that ovulated did so on the second or third night following commencement of the temperature increase. In fish that ovulated, plasma GtH surged in the afternoon, reached a peak at midnight, then returned to basal levels by morning. In some of the ovulated fish, a gradual increase in plasma GtH was observed at initiation of the temperature increase. Ovulation took place after the GtH surge commenced, at midnight or early in the morning. Spawning took place immediately after ovulation. T increased when the temperature was first elevated and remained at elevated levels during the GtH surge, while plasma E2 rose only slightly. Both E2 and T returned to basal levels on the morning after spawning. 17,20-P levels varied with GtH levels, reaching

a peak at approximately midnight and decreasing thereafter. 17-P increased significantly in the afternoon prior to ovulation and peaked before GtH peaked in the majority of the fish. Because 17-P levels increased before GtH and 17,20-P, which increased together, it was hypothesized that GtH induced synthesis of 17,20-P from precursor 17-P (Santos et al., 1986). In females that did not ovulate, no large peaks of plasma GtH or steroid hormones were observed (Santos et al., 1986). Females that ovulated on the second or third night demonstrated the preovulatory GtH surge each evening, indicating that this surge is induced by photoperiod. The GtH surge occurs in the latter part of the light phase following the temperature increase irrespective of the time of day when the temperature rise is initiated (Santos et al., 1986). It appears that temperature, steroid hormone levels, and possibly other factors prime the system for the GtH surge, and photoperiod controls the time of day when ovulation takes place. Low plasma E2 levels indicate that vitellogenesis was probably already completed prior to the study (Santos et al., 1986).

Spawning

Spawning in wild carp is controlled by factors such as temperature, photoperiod, presence of a suitable spawning substrate such as aquatic vegetation, and proximity of reproductively ready potential mates (Billard, 1999a). Carp generally will not spawn when held at temperatures of 16 °C

(Billard, 1999a) or less and are not known to spawn at all below 15 °C (Table 1-3). Oogenesis, or oocyte development, does not occur below 15 °C (Horvath, 1986). Ovulation can be induced artificially by applying a temperature shock (heat hypophysation) in which water temperature is increased from 16 to 24 °C in a period of approximately 6 to 8 hr beginning in late afternoon (Billard, 1999a). GtH increases at the end of the following day, and ovulation occurs around midnight of the following night (Billard, 1999a). Spermiation in males apparently is induced by pheromones released by ripe females (Billard, 1999a). It is thought that the pre-spawning GtH surge necessary for spermiation in male carp might be induced by pheromones released by female carp undergoing final oocyte maturation (Barry et al., 1990a; Barry et al., 1990b). In the closely related goldfish, it is well-established that ovulatory females release one or more pheromones that stimulate the GtH surge and spawning behavior in males (Kobayashi et al., 1986; Dulka et al., 1987; Sorensen et al., 1988; Van Der Kraak et al., 1989).

Hormone changes during ovulation in common carp and goldfish have been described (Aida, 1988). When these fish were allowed to spawn naturally, a preovulatory GtH surge took place during the latter half of the light phase. GtH levels peaked at around midnight, when ovulation took place. Ovulation usually took place during the dark phase, regardless of manner in which the

temperature and photoperiod were manipulated, indicating that the GtH surge is triggered by a photoperiodic cue. T, 17-P, and 17,20-P increased coincident with the GtH increase. E2 levels increased slightly during spawning in goldfish, but remained at the same low level in carp. In male fish, an increase in water temperature resulted in a slight rise in GtH when females were not present. The presence of ovulatory females caused a significant increase in GtH levels in the blood of males. This effect of females on males appears to be mediated by pheromones and ensures that ovulation and production of milt occur together so that fertilization is likely to be achieved (Aida, 1988).

Two-cell theory of steroidogenesis

Female

A two-cell theory for ovarian steroidogenesis of E2 and maturation-inducing hormone has been described (Nagahama, 1987). Briefly, during vitellogenesis and oocyte growth, GtH stimulates thecal cells of an ovarian follicle to produce the androgen testosterone (T). T diffuses into the granulosa cell layer where GtH stimulates the enzyme aromatase to convert T to E2. During oocyte maturation in preparation for spawning, GtH stimulates the thecal cells to produce 17α-hydroxyprogesterone (17-P), which crosses the basal lamina to the granulosa cells. GtH stimulates the

enzyme 20β-hydroxysteroid dehydrogenase in the granulosa cells to convert 17-P to 17,20-P, which induces final oocyte maturation.

Male

In the testis, Leydig cells are the steroidogenic cells. Mature spermatozoa have 20β-hydroxysteroid dehydrogenase activity and convert 17-P to 17,20-P just prior to spermiation (Barry et al., 1990a).

Gonadotropins and gonadotropin-releasing hormone

It is now generally accepted that common carp and goldfish (and many other species of teleostean fish) have two gonadotropins. Because it was previously believed that there was only one, much of the literature refers to a single gonadotropin. Since the discovery of more than one, it has been demonstrated that there is considerable overlap between the two gonadotropins in their ability to stimulate ovarian and testicular steroidogenesis and final oocyte maturation, particularly in carp (Van Der Kraak et al., 1992). It is likely that the difference in timing of their release is responsible in part for the differences in their functions. Gonadotropin I (GtH-I) is the approximate equivalent of follicle stimulating hormone (FSH) in mammals. Gonadotropin II (GtH-II) is the approximate equivalent of leutenizing hormone (LH) in mammals. For the purposes of this review, the term GtH without a designation of I or II is used when the study was

conducted under the assumption that there was only one gonadotropin, unless a distinction can be made with regard to the specific GtH under discussion. Two gonadotropins, GtH-I and GtH-II, have been purified from the pituitary of female common carp and characterized. GtH-I and GtH-II have molecular weights of 45,000 and 35,000, respectively (Van Der Kraak et al., 1992). Like mammalian GtHs, carp GtHs each consist of two subunits, α and β. Results of testing with antisera to the α-subunit of a maturational GtH suggest that both GtHs have the same α-subunit (Van Der Kraak et al., 1992), as is the case with mammalian GtHs (Norris, 1997b). At least four types of gonadotropin-releasing hormone (GnRH) are found in osteichthyan (bony) fishes, and two of these have been identified in the hypothalamus and pituitary of goldfish (Redding et al., 1993).

Factors affecting plasma sex steroid hormone concentrations

Temperature

Temperature control of testicular steroidogenesis was illustrated by incubation of goldfish testis *in vitro* with T (Kime, 1979). Conversion of T to 11-KT increased with temperature, reached a plateau at 11 °C, and decreased above 36 °C. The effect of temperature appeared to be permissive rather than regulatory. The plateaus for formation of testosterone-glucuronide (T-gluc) and 11-ketotestosterone glucuronide (11-KT-gluc) occurred at a greater temperature than the plateau for conversion of

T to 11-KT. The plateau of 11-KT synthesis is a result of conversion of 11-KT to 11-KT-gluc and decreased availability of substrate T due to formation of T-gluc. Glucuronidation of T and 11-KT increases with temperature, resulting in decreased output of 11-KT. The range of maximum 11-KT output corresponds to optimum spawning temperatures for goldfish.

Temperature also is known to have a pronounced effect on ovarian steroidogenesis in carp (Manning et al., 1984). As in goldfish testis, glucuronyl transferase activity in the carp ovary increases with temperature, with the most significant change in activity occurring between 20 and 24 °C, a range of temperatures increasingly favorable for reproduction in carp (Manning et al., 1984).

Peripheral metabolism

Blood cells of goldfish have 17β-hydroxysteroid dehydrogenase activity, and thus are capable of converting 11-ketoandrostenedione to 11-KT (Mayer et al., 1990). Conversion of 11-ketoandrostenedione to 11-KT in the peripheral circulation might be important for the maintenance of appropriate blood levels of the most biologically active androgens in fish (Mayer et al., 1990).

Stress

It has been speculated that stress could cause an increase in 11-KT synthesis, at least in migratory salmon, because cortisol can be converted by fish liver to androstenedione, which is in turn readily converted to 11-KT in the testis (Kime, 1979). Also, stress is known to cause signficant reductions in concentrations of circulating sex steroids in fish within a few hours of stressor introduction (McMaster et al., 1992). This is a particularly important consideration when plasma sex steroid concentrations are used as endpoints in reproductive toxicity testing, since capture, handling, etc., all are stressful experiences for fish.

Exposure to environmental pollutants

Exposure to certain environmental pollutants can alter plasma sex steroid concentrations indirectly by increasing metabolism and clearance. For example, intraperitoneal injection of polychlorinated biphenyls (PCBs) in adult male and female carp increased metabolism of progesterone (P), E2, and T by hepatopancreas microsomes and caused a significant reduction in the circulating concentrations of P and E2 in females and T in males (Yano et al., 1986). The decreases in circulating concentrations of these steroids were probably due to increased metabolism by the hepatopancreas (Yano et al., 1986).

Normal plasma sex steroid concentrations

In a study conducted in Japan, concentrations of plasma sex steroid hormones were measured in adult common carp held at 18 to 20 °C under a 12L/12D photoregime for 2 wk. The plasma concentrations of sex steroids were reported as the mean for 10 fish ± SD. In female carp, the plasma progesterone (P) concentration was 126 ± 41 pg/mL and plasma E2 concentration was 62 ± 23 pg/mL. In male carp, the plasma T concentration was 1.79 ± 0.82 ng/mL.

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Table 1-1. Gonadosomatic indices (GSI) for female common carp

GSI	Reference
immature ovaries 0.8%, maturing 0.8- 2.0%, advanced maturing 2.0- 8.0%, mature ≥ 8.0%, ripe up to 20% of total body weight	Gupta, 1975
fully mature ovaries were 3.67- 37.93% of total body weight, average 19.59%; varied with geographic region	Parameswaran et al., 1972
maturing to ripe ovaries ranged from 1.93- 14.4% of total body weight	Manning and Kime, 1984
post-spawning ovaries averaged 3.9% (2.3- 7.5%) total body weight, increased to an average of 11.8% (5.8- 17.8%) during recrudescence, rose to an average of 17.6% (8.3- 38.5%) prior to spawning	Shikhshabekov, 1972
juvenile mirror carp GSI approximately 1% total body weight, mature mirror carp GSI up to 27%	Tyler and Sumpter, 1990
GSI = 0.86% ± 0.12 total body weight for immature females, 5.72% ± 0.47 post-spawning, approximately 28% in mature females	Crivelli, 1981
0.9- 11.4% total body weight (increased with age from 2- to 5-yr-old carp)	Horvath, 1986

Table 1-2. Gonadosomatic indices (GSI) for male common carp

GSI	Reference
mature testes weighed 5% of total body weight	Gupta, 1975
fully mature testes were 1.76- 30.36% total body weight, average 13.99%; varied with geographic region	Parameswaran et al., 1972
post-spawning testes weighed an average of approximately 2% total body weight, GSI increased to approximately 18.5% in mature males	Guha and Mukherjee, 1987
juvenile mirror carp GSI < 0.3% total body weight, mature male mirror carp GSI >5%	Tyler and Sumpter, 1990
GSI of fully mature males was 14.0 - 21.8%	Huang and Chang, 1980
mature male GSI post-spawning approximately 4.5% total body weight, maximum GSI >8%	Crivelli, 1981
testes averaged 5.7% (3.5- 10.4%) of total body weight during recrudescence and reached an average of 6.8% (3.3- 14.4%) in mature males	Shikhshabekov, 1972

Table 1-3. Spawning temperatures for common carp in selected locations

Temperature	Location	Reference
>17 °C, optimum 19- 23 °C, decreased at >26 °C, stopped at 28°C	Lake St. Lawrence, Ontario	Boon Swee and McCrimmon, 1966
optimum at 24 °C	United Kingdom	Manning and Kime, 1984
minimum temperature of 17 °C	Arakum Lakes, Dagestan	Shikhshabekov, 1972
18- 22 °C, decreased at >26 °C, stopped at 28 °C; no oogenesis below 15 °C		Horvath, 1986

Figure 1-1. Diagram of the hypothalamic-pituitary-gonad axis (HPG-axis) in teleost fish. GtH-I = gonadotropin I, GtH-II = gonadotropin II, GnRH = gonadotropin-releasing hormone.

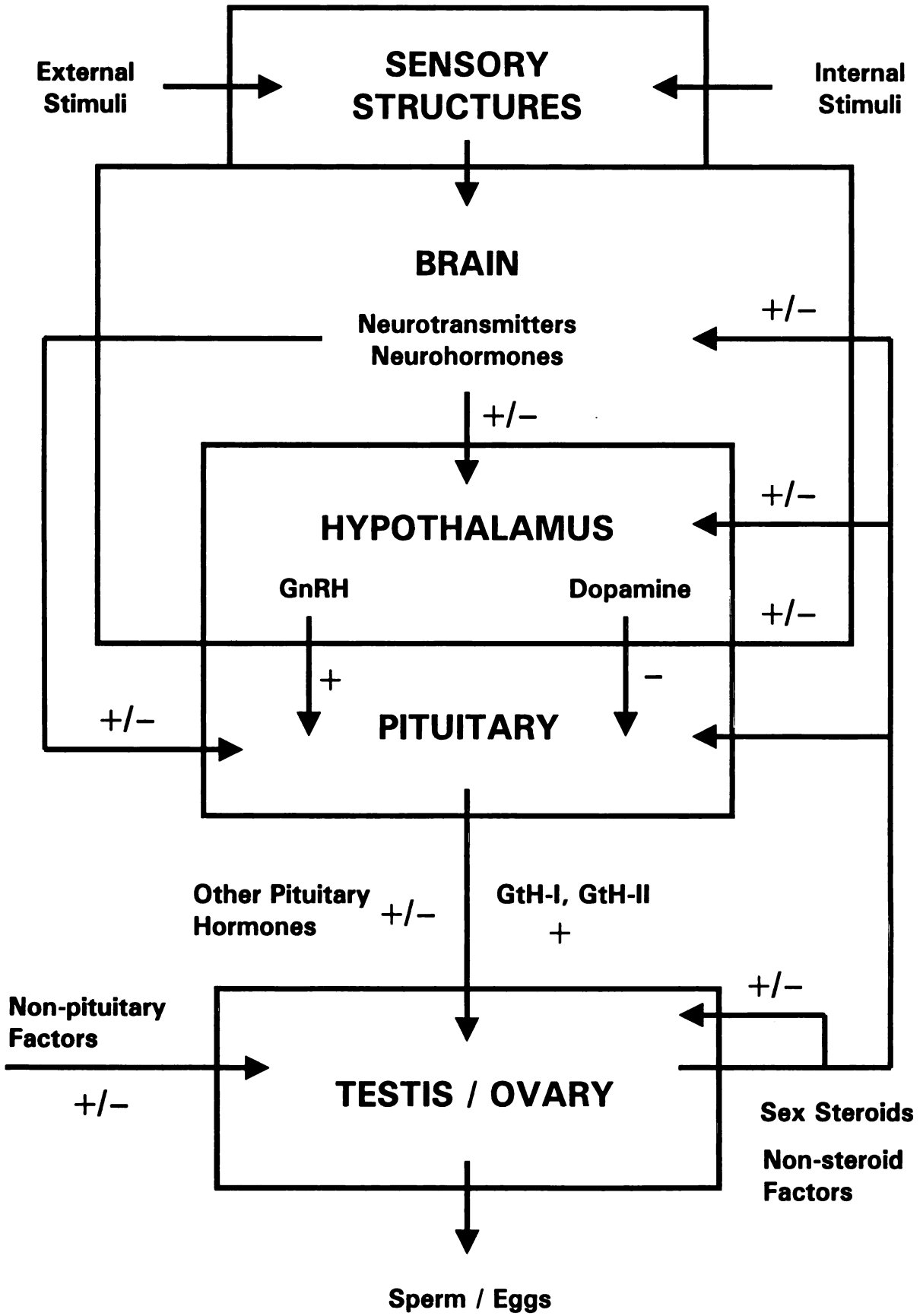
External Stimuli
SENSORY STRUCTURES
Internal Stimuli
BRAIN
Neurotransmitters
Neurohormones
+/−
+/−
HYPOTHALAMUS
+/−
GnRH
Dopamine
+/−
+
−
+/−
PITUITARY
Other Pituitary Hormones
+/−
GtH-I, GtH-II
+
+/−
Non-pituitary Factors
+/−
TESTIS / OVARY
Sex Steroids
Non-steroid Factors
Sperm / Eggs

Figure 1-2. Pathway of steroidogenesis in fish ovary. 3β-HSD = 3β-hydroxysteroid dehydrogenase, 20β-HSD = 20β-hydroxysteroid dehydrogenase, 17β-HSD = 17β-hydroxysteroid dehydrogenase, 11β-HSD = 11β-hydroxysteroid dehydrogenase, SCC = cholesterol side chain cleavage enzyme. Taken from Nagahama (1987), description of steroidogenesis in salmonid ovary. * Enzymes responsible for conversion of testosterone to 11-ketotestosterone in the goldfish testis (Barry et al., 1989).

Oocyte maturation
Vitellogenesis
Cholesterol
SCC
Pregnenolone
3β-HSD
Progesterone
20β-HSD
17α,20β-Dihydroxy-4-pregnen-3-one
17α-Hydroxyprogesterone
C17-20lyase
Androstenedione
17β-HSD
Testosterone
Aromatase
17β-Estradiol
11β-hydroxylase or 11β-HSD*
11-Ketotestosterone

Chapter 2

EVALUATION OF REPRODUCTIVE ENDPOINTS IN COMMON CARP (*CYPRINUS CARPIO*) CAGED *IN SITU* AT SITES RECEIVING WASTEWATER FLOW IN LAKE MEAD, NEVADA, (MARCH TO APRIL)

(Intended for submission to *Aquatic Toxicology*)

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ABSTRACT

Recent reports have indicated that chemical contaminants entering Lake Mead, Nevada, via the Las Vegas Wash are capable of causing estrogenic effects in wild common carp and in an estrogen-responsive cell bioassay. Adult male and female common carp (*Cyprinus carpio*) were exposed separately in cages (30 fish per cage) for 42 to 48 d at four sites in Lake Mead: one site directly in the influent of the Las Vegas Wash as it enters Lake Mead, one site in Las Vegas Bay where the influent of the Wash is more dilute, and two reference sites. Endpoints examined included condition factor (K), gonadosomatic index (GSI), plasma concentrations of sex steroids and vitellogenin (VTG), and histology of the hepatopancreas, ovary, and testis. Plasma sex steroids measured were 17β-estradiol (E2), testosterone (T), and 11-ketotestosterone (11-KT). There was no effect of Las Vegas Wash influent on GSI, ovarian follicle development, hepatopancreas histology, or gonad histology. Although Las Vegas Wash influent might have altered steroid hormone profiles in the carp, it could not be determined conclusively that the effects were due to chemical exposure rather than site-to-site temperature variation. The median plasma VTG concentration in male carp caged in the Las Vegas Wash was 3- to 10-fold greater than concentrations measured in male carp at all other sites. Although there was an increase in plasma E2 concomitant with the increase in plasma VTG in male carp caged in the Las Vegas Wash, similar increases in plasma E2 did

not result in VTG induction in male fish caged at some of the other sites. This indicated that the male carp caged in the Las Vegas Wash possibly had been exposed to an exogenous estrogenic chemical. However, an effect of site temperature on plasma sex steroid levels subsequently resulting in VTG induction cannot be discounted as a potential cause.

INTRODUCTION

Lake Mead is a large reservoir formed by impoundment of the Colorado River behind the Hoover Dam. The reservoir serves as a source of domestic and agricultural water for more than 22 million users (LaBounty et al., 1997) and, as part of the Lake Mead National Recreational Area, is used for activities such as boating, swimming, and fishing. The Las Vegas Wash enters the Boulder Basin of Lake Mead via the Las Vegas Bay (Figure 2-1) and provides one the greatest inflows of water into Lake Mead, second only to the Colorado River (LaBounty et al., 1997). The flow of the Las Vegas Wash consists entirely of all tertiary treated municipal sewage effluent, urban storm water flow, and groundwater seepage from the urbanized Las Vegas Valley (LaBounty et al., 1997). The water entering Lake Mead via the Las Vegas Wash is considerably more dense and exhibits greater conductivity and turbidity than main body Lake Mead water (LaBounty et al., 1997). The difference in density causes the intrusion of the Las Vegas Wash to form an interflow that extends from Las Vegas Bay into the Boulder Basin. At times,

the influence of this interflow has been detected on the basis of its conductivity at the Hoover Dam, 16 km away from the confluence of the Las Vegas Wash with Lake Mead.

In 1996, the United States Geological Survey (USGS) reported evidence that suggested endocrine disruption in wild adult male and female common carp (*Cyprinus carpio*) associated with the Las Vegas Wash and Bay (Bevans et al., 1996). This evidence included alterations in plasma sex steroid concentrations in male and female fish relative to those observed in fish at a reference site and induction of a female-specific protein, vitellogenin (VTG), in the blood of male carp in the absence of a concomitant increase in plasma 17β-estradiol. These findings prompted concern for the razorback sucker (*Xyrauchen texanus*), an endangered species of fish that spawns in the Las Vegas Bay (Holden et al., 1999).

The potential causes of the effects observed in the Lake Mead carp are many, but the induction of VTG in male fish without a concomitant increase in plasma E2 indicated that the fish had been exposed to an estrogen-like (estrogenic) chemical in the environment. VTG is an egg yolk precursor synthesized in the liver of fish in response to the estrogenic sex steroid 17β-estradiol (E2). VTG is transported in the blood to the ovary, where it is sequestered into developing oocytes in preparation for spawning (Mommsen

et al., 1988). VTG serves no known function in male fish and normally is not detected (or detected only in very low concentrations) in their blood plasma, presumably because E2 levels are not great enough in male fish to induce VTG synthesis (Mommsen et al., 1988; Specker et al., 1994). However, male fish possess the VTG gene and are capable of producing VTG when exposed to exogenous estrogenic chemicals (Mommsen et al., 1988; Specker et al., 1994). VTG is a useful biomarker of exposure to estrogenic chemicals in male fish for several reasons. VTG induction is a specific response to estrogenic chemicals, and, in male fish, is attributable to exposure to an estrogenic substance (Mommsen et al., 1988). Because male fish do not possess a normal clearance mechanism for VTG (i.e., deposition into oocytes), it can accumulate to considerable concentrations in their blood plasma (Mommsen et al., 1988). Also, only a small volume of plasma is needed for measurement of VTG, leaving the remainder for measurement of plasma sex steroids or other hormones that might be important in evaluating potential reproductive dysfunction.

Exposure to an estrogenic chemical also might cause alterations in plasma sex steroid levels in fish, although not necessarily through an estrogen receptor-mediated mechanism (MacLatchy et al., 1997; Tremblay et al., 1998). Given that the Las Vegas Wash consists of wastewater, which can be expected to contain a complex mixture of chemical contaminants, the

effects on sex steroids and on plasma VTG in Lake Mead carp might have occurred through entirely different mechanisms and/or by exposure to different chemicals. Studies in the United Kingdom (UK) (Harries et al., 1996; Harries et al., 1997; Harries et al., 1999) and in the United States (US) (Folmar et al., 1996) on fish in municipal sewage effluent or in rivers receiving it have demonstrated that contaminants in sewage effluent can cause VTG induction and other reproductive effects in fish. Results of the work conducted in the UK indicated that VTG induction observed in fish in sewage effluents and in some rivers might be caused by water soluble contaminants such as the animal steroids E2 and estrone (E1) and/or an oral birth control medication component, ethinylestradiol (EE2) (Routledge et al., 1998).

To investigate the possibility that water soluble contaminants in the Las Vegas Wash might be causing estrogenic effects in Lake Mead fish, researchers from Michigan State University (MSU) adopted a toxicity identification and evaluation (TIE) scheme to accomplish two goals initially: (1) to determine whether extracts from water samples collected from the Las Vegas Wash (LW) and Las Vegas Bay (LX) were capable of producing an estrogenic response *in vitro* in MCF7-luciferase cells, and (2) to identify likely chemical causes for any *in vitro* responses observed. In 1997, 5 L water samples were collected from LW, LX, and two reference sites in Lake Mead

(Saddle Island and Callville Bay) and subjected to solid phase extraction (Snyder et al., 1999). The resulting extracts were fractionated into three classes based on polarity, and each class was screened for estrogenic activity using an *in vitro* bioassay (MCF7-luciferase) (Snyder et al., 2000). These same fractions were analyzed for the presence of selected estrogenic chemicals by separation of analytes with high-pressure liquid chromatography (HPLC) and subsequent detection by fluorescence or radioimmunoassay (RIA). Significant estrogenic responses were induced in the bioassay by the extracts from LW and LX and not by extracts from the reference sites. The bioactivity of these extracts was associated with the fraction of greatest polarity. Mass-balance analysis of known estrogenic chemicals identified in the fractions indicated that steroidal estrogens such as E2 and EE2 were likely causes for the observed bioactivity of the LW and LX extracts. Although they have been implicated as potential causative agents for endocrine disruption in fish exposed to sewage effluent in the UK (Purdom et al., 1994; Harries et al., 1997), alkylphenol ethoxylates and their degradation products, the alkylphenols, are not likely to have contributed significantly to the estrogenic bioactivity of these Lake Mead water extracts *in vitro* (Snyder et al., 2000).

Although steroidal estrogens appeared to be the most potent estrogenic chemicals in extracts from LW and LX when tested *in vitro*, it cannot be

assumed that this would hold true *in vivo* as well. Estrogens produce a variety of effects at multiple targets, some of which involve interaction of different tissues; these types of effects cannot be predicted with a single *in vitro* bioassay (Zacharewski, 1997). *In vitro* bioassays also do not account for bioaccumulation or metabolism (Zacharewski, 1997), which can cause substantial differences in the responses of whole animals versus cells in culture. Therefore, the study reported here was conducted to address the following questions: (1) Can effects observed in wild carp by researchers at USGS be reproduced in caged fish? (2) Are steroidal estrogens and other relatively polar contaminants present in the Las Vegas Wash and Las Vegas Bay at concentrations likely to cause reproductive effects observed in fish? For several reasons, this study complements the ongoing USGS work with wild carp. The wild carp represent fish receiving natural exposures. Wild carp will feed on detritus in the lake sediment and might be exposed to more hydrophobic chemicals present in food or sediment by this route. They also might receive longer-term exposure or exposure during critical periods of development that might result in effects that would not be detected with a short-term exposure conducted with adult fish. However, the caged fish receive a more controlled exposure with a specified duration and under monitored conditions. Wild fish are free to move in and out of the influence of the Las Vegas Wash, whereas caged fish receive a known level of exposure.

METHODS AND MATERIALS

Fish

Sexually mature, adult male and female common carp (*Cyprinus carpio*, 2 to 3 yr of age) were purchased from J&J Aquafarms (Sanger, California). Male and female carp were held in separate cement holding ponds at the Lake Mead Fish Hatchery, Boulder City, Nevada, for the shortest length of time possible (4 to 7 d) prior to placing them into cages in the field. Although a longer acclimation period was preferable, outbreaks of *Ichthyophthirius multifilis* ("Ich") in the hatchery fish are common even when fish are stocked under optimal conditions, and a longer acclimation period might have required prophylactic treatment with chemicals that could affect the endpoints of interest. While fish were held at the hatchery, they were fed daily to satiation with a floating dense culture pellet food for pond fish (Aquatic Ecosystems, Apopka, Florida; F2G). A floating pellet formulation was used so that fish could be observed at the surface for normal feeding behavior and health. Fish were not fed the day prior to placing them in cages.

Cage deployment

Fish cage kits were purchased from Aquatic Ecosystems (Apopka, Florida; catalog number C3). Cages were constructed of a polyvinyl chloride (PVC) pipe frame and polypropylene knotless mesh in order to prevent injury to the

fish. Cage dimensions were 2.1 m X 2.1 m X 1.4 m deep. Cages were suspended approximately 1.4 to 1.7 m below the surface of the water to minimize the effects of wave action, interference with boating and other recreational activities, and possibility of vandalism or disturbance to the fish. Because there were no surface objects to support the cages, they were suspended by attaching the lower frame of the cage with cables to an anchor on the lake bottom. The cages were vertically collapsible, so floats attached to the top of the cage cause it to rise toward the surface and prevented collapse of the cage on the fish. All sides of the cages were constructed of mesh to maximize water movement through the cages, to facilitate feeding and observation, and to allow waste to drop through.

Two cages, one containing 30 males and one containing 30 females, were deployed at each of four sites in Lake Mead (Figure 2-1). Two sites, Water Barge Cove (WB) and Moon Cove (MC), are not influenced significantly by the flow of the Las Vegas Wash and could be considered reference sites. These reference sites also were close to the reference sites used in the 1997 examination of water extracts by analytical chemistry and *in vitro* bioassay techniques. Callville Bay, not labeled on the map, is just adjacent to Water Barge Cove. The Saddle Island reference site from the 1997 study is near Moon Cove. Two cages were placed directly at the point where the Las Vegas Wash enters Las Vegas Bay such that the carp caged at this site

received maximum exposure to the influent. This site was named Las Vegas Wash (LW). The remaining two cages were placed further out in Las Vegas Bay at a point where the flow entering from the Wash was more dilute but still readily detected by its greater conductivity and turbidity than main body lake water. All sites were monitored for conductivity and other water quality parameters (see below), in part to ensure that the fish were receiving the expected exposure to the flow of the wash. Following placement of cages in the field, some carp were retained at the Lake Mead Fish Hatchery (HF).

Initial total weight of carp placed in each cage was determined by weighing a bucket of water on a scale, then adding the fish and re-weighing. The initial mean weight was calculated by dividing the initial total weight by the number of fish weighed and converting to mean weight in grams per fish. Fish were transported by boat in aerated livewells to the exposure sites.

Exposure site monitoring

Fish cages at the reference sites were readily observed from the surface, but often could not be seen from the surface at sites LX and LW due to greater water turbidity, so the cages at the latter two sites were marked with surface buoys. The depth of the lake rose and fell occasionally due to water releases at the Hoover Dam, so cage depth was checked during site visits and the depth re-adjusted as necessary. Fish at the reference sites also were

checked by divers to ensure that the fish were not suffering obvious damage or stress. Divers could not check the carp caged at LX and LW because body contact with the water at those sites is not recommended and visibility is low. Fish were fed approximately twice weekly or as weather permitted by dropping a sinking pellet food (Silver Cup trout pellets; Nelson & Sons, Inc.; Murray, Utah) through the top of each cage. Fish were observed to be feeding at the reference sites but could not be seen at sites LX and LW.

Water quality

Water temperature, pH, dissolved oxygen (DO), conductivity (expressed as microSiemens), and turbidity were measured using a Hydrolab Surveyor 4™ data recorder equipped with a H_2O Sonde™ water quality multiprobe (Hydrolab Corporation, Austin, Texas). Measurements taken at 1, 2, and 3 meter depths were averaged to cover the entire span of the fish cages. Water samples for measurement of alkalinity, hardness, total ammonia, unionized ammonia, and nitrite were collected at approximately mid-depth of the cages at each site with a horizontal water sampler (Wildlife Supply Company, Saginaw, Michigan, catalog number 7510-C22), and the water quality parameters were measured by use of Hach test kits (Hach Company, Loveland, Colorado; catalog numbers 20637-00, 20636-00, 2241-00, 2240-00).

Cage retrieval and sample collection

Two fish cages (one for each sex) were retrieved from one site per sampling day. Cage retrievals at the different sites were conducted over the shortest period of time possible to minimize variation in the endpoints of interest. Because a boat was required to retrieve the cages, poor weather prevented sampling on a daily basis. Lack of space on the boat and wave action prevented safe handling of needles on the boat. Therefore, fish were collected from the cages and placed into aerated livewells for transport a short distance back to a dock for sampling. At the dock, fish were killed by overdose of approximately 200 mg/L tricaine methanesulfonate (Tricaine-S or MS-222; Aquatic Ecosystems, Apopka, Florida; TRS1). Pressure was applied to the abdomen of male fish to determine whether milt was present. Blood was collected immediately from the caudal vasculature of each fish using a chilled 3 mL heparinized syringe fitted with a heparinized 20- or 22-gauge X 1.5 inch needle. Up to 3 mL blood was placed into chilled polyethylene centrifuge tubes pretreated with 3 TIU aprotinin (Sigma, St. Louis, Missouri; A-1153) in 30 µL solution (0.9% sodium chloride; 0.9% benzyl alcohol), for a final concentration of ≥ 1 TIU aprotinin per mL blood. Aprotinin is a protease inhibitor used to prevent the breakdown of VTG. The tubes were gently shaken by hand to mix the blood and aprotinin solution, then placed on ice for transport back to the laboratory. Each fish was quickly examined for external injuries, external parasites, and obvious gill

damage, then weighed to the nearest 1 g and the length measured to the nearest 0.1 cm. Each carcass was placed into an individual labeled bag and transported on ice back to the laboratory for dissection. In order to minimize variation in plasma hormone levels caused by diel cycling, fish of the same sex at different sites were consistently sampled for blood within the same 2- to 3-hr time period (McMaster et al., 1992).

In the laboratory, the abdominal cavity of each fish was opened to confirm the sex by observation of the gonads. In the interests of time and obtaining fresh samples, a random subsample of 16 fish from each cage per site (16 fish of each sex) was selected for collection of gonad weights and histology samples. Gonads were removed and weighed to the nearest 0.001 g with a portable analytical balance (Acculab V-1mg, Newtown, Pennsylvania) for calculation of gonadosomatic index (GSI), where GSI = [(gonad weight)/ (total body weight - gonad weight)] and all weights are in g. One gonad was snap-frozen in liquid nitrogen and stored at -80 °C for future analyses. The caudal half of one gonad from each fish was preserved in neutral-buffered formalin for later histological examination of sections taken from the centermost portion of the gonad. The hepatopancreas was removed and a small portion fixed in neutral-buffered formalin for histological examination. The remainder of the hepatopancreas was split into two cryotubes and snap-frozen in liquid nitrogen, then stored in liquid nitrogen for future analyses.

At the laboratory, blood samples were centrifuged at 3000 × g for 10 min at 4 °C. Plasma was collected, divided into several aliquots per sample, and frozen at -80 °C. Portions of each plasma sample were frozen separately for sex steroid and VTG analysis such that samples would not be subjected to repeated freezing and thawing. VTG is particularly sensitive to repeated freezing and thawing, so multiple tubes per sample were reserved for VTG analysis.

Final total weight was calculated by summing the individual weights of fish recorded when the fish were retrieved and dividing by the number of fish retrieved. Because the method of measurement of initial weight was crude and the measurements of initial and final weights were made in different fashions, comparisons between initial and final weights should be considered as rough estimates. These measurements were made only to determine whether fish weight, and thus presumably condition, was reduced by stress related to caging. Individual measurements of weight and length were not made on fish prior to caging because it was necessary to reduce handling stress to the greatest extent feasible. Likewise, weighing the fish as a group at the end of the exposure to obtain a measure analagous to the initial weight determination would have increased handling stress and slowed sample collection during a critical time period. Fulton-type condition factors (K) were calculated from length and weight measurements as $K = (W/L^3) \times$

10,000 where W= weight in g and L= length in mm (Anderson et al., 1996).

The excess carp remaining at the Fish Hatchery (HF) also were sampled in the same fashion as the carp caged in the field. However, it was not possible to hold the carp at HF until the end of the field study, so they were sampled 14 d after the last pair of cages was deployed in the field. Because these fish were not held under the same conditions or for the same length of time as the carp caged in the field, the carp held at HF were not considered to be comparable to the caged fish. The carp held at HF were sampled only for any potentially useful information they might yield concerning background concentrations of plasma VTG or state of sexual development of the fish at the beginning of the study.

Histology

Gonad and hepatopancreas samples taken for histological examination were fixed in neutral-buffered formalin. Sections from the centermost portion of each gonad were trimmed and placed into tissue cassettes. Ovaries were sectioned transversely and testes were sectioned longitudinally. Trimmed tissues were placed into tissue cassettes, and submitted to the Histology Laboratory at the Michigan State University Clinical Center for processing. Tissues were embedded in paraffin, sectioned at 5 μm, and stained with

haematoxylin and eosin. All slides were examined by a Board-Certified (American College of Veterinary Pathologists) veterinary pathologist.

Hepatopancreas histologic criteria

Slides of hepatopancreas tissue were examined for signs of necrosis, inflammation, neoplasia, foci of atypia, biliary stasis, and cellular degeneration. Vacuolar degeneration of hepatocellular cytoplasm (hepatocellular vacuolation) is a nonspecific lesion and indicates accumulation of glycogen and/or fat due to overfeeding, emaciation (use of fat stores), toxification due to various substances, and other causes. Hepatocellular vacuolation was graded on a scale as follows: 0 = no vacuolation, 1 = mild vacuolation with small vacuoles spread throughout the cytoplasm, 2 = moderate vacuolation with larger coalescing vacuoles appearing as large clear zones in many hepatocytes, 3 = severe vacuolation where all or most of the cytoplasm has lost its normal pink coloration due to confluent, large, clear vacuoles.

Ovary histologic criteria

Stages of ovarian follicle development were assessed as described previously (Table 2-1) (Miles-Richardson et al., 1999b). A typical area of the ovary was selected, and 50 follicles were counted within that area. Proportions of primary, secondary, tertiary, and atretic follicles per 50 follicles are recorded.

Atretic follicles are unovulated follicles undergoing atresia, a process of degeneration.

Testis histologic criteria

Sections of testis were scanned at 50X and 100X magnifications, and the relative number and prominence of Sertoli cells in comparison to control fish were estimated using the following scale: 0 = no proliferation, 1 = mild proliferation (<one third), 2 = moderate proliferation (one third to two thirds), 3 = severe proliferation (> two thirds). Sertoli cell proliferation previously was observed in male fathead minnows exposed to the natural estrogen 17β-estradiol (E2) or to the estrogenic chemical 4-nonylphenol (Miles-Richardson et al., 1999a; Miles-Richardson et al., 1999b). Sections of testis were examined for the presence of degenerative changes such as germ cell syncytia, mineralization of spermatozoa, and variably sized or necrotic spermatozoa (Miles-Richardson et al., 1999a; Miles-Richardson et al., 1999b). Testes also were designated as spermatogenically active or inactive.

Plasma vitellogenin (VTG)

Plasma samples were analyzed for VTG with an competitive enzyme-linked immunosorbent assay (ELISA) technique previously developed at Michigan State University (Nichols et al., 2000) for use with goldfish and fathead

minnow plasma samples. Although the original ELISA, which was developed with goldfish VTG as a standard, functioned well as a heterologous ELISA for fathead minnow VTG, it did not perform well for measurement of carp VTG, possibly due to the use of a different lot of primary antiserum. Modifications, including the use of purified carp VTG as a standard, were made to the original procedure for measurement of VTG in common carp blood plasma.

VTG induction in carp

Male common carp were injected with 17β-estradiol (E2) (Sigma, St. Louis, Missouri; E-8875) at a rate of 10 mg E2 • kg body weight^{-1} • wk^{-1} for two wk (2 injections per fish) to induce VTG synthesis. E2 was dissolved in 100 μL 70% ethanol, then mixed with 100 μL sterile 0.9% sodium chloride (NaCl). Fish were anesthetized in a solution of 50 mg/L tricaine methanesulfonate buffered to pH 7.0, and the E2 solution was administered by intraperitoneal injection with a 1 mL sterile syringe and sterile 25 gauge X 1 inch needle. Seven days after the last injection, one fish was anesthetized as described previously and given a single 100 μL intraperitoneal injection of 10% aprotinin in sterile 0.9% NaCl using a sterile syringe and needle. Thirty min later, the same fish was killed by overdose of tricaine methanesulfonate (200 mg/L), and the blood was collected in the same manner as described previously for fish caged in the field. Blood was centrifuged at 3000 × g for

10 min at 4 °C. The plasma was removed and frozen in working aliquots at -80 °C until needed.

VTG purification

VTG was purified from carp plasma by high-pressure liquid chromatography (HPLC) using a method described previously (Silversand et al., 1989; Nichols et al., 2000), with a few modifications. Briefly, 1 mL of plasma from the E2-induced carp was thawed, filtered with a 0.2 μm Gelman syringe filter and diluted in 9.0 mL of filtered 20 mM Tris-HCl buffer (Buffer A: pH 8.0, 0.1% aprotinin solution (7.6 TIU/mL, Sigma, St. Louis, Missouri; A-6012)). The diluted plasma (500 μL) was injected onto a DEAE 5 PW anion exchange column (7.5 X 150 mm; Toyo-Soda; PJ Cobert Assoc., St. Louis, Missouri) equilibrated with Buffer A. Following an initial wash with Buffer A to flush out unbound material, proteins bound to the column were eluted with a linear gradient of 100% Buffer A to 100% Buffer B (0.5 M NaCl in Buffer A) for 50 min at a flow rate of 1.0 mL/min. The method was improved by introducing a backward gradient at 25 min from 50% A, 50% B to 57% A, 43% B over 2 min to increase the resolution of VTG from other plasma proteins. From 27 min, the linear gradient to 100% B was resumed. Eluted proteins were monitored by ultraviolet (UV) fluorescence with a photodiode array fluorescence detector. Absorption was measured at 230, 254, and 280 nm.

VTG separation and identification

The HPLC fraction believed to be VTG was collected from two HPLC separations, mixed, and frozen in 10 μL aliquots at -80 °C for later use as a standard in the VTG ELISA. A larger portion was reserved for confirmation of the presence of carp VTG by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting. The putative purified carp VTG along with goldfish VTG purified in the same manner were run simultaneously and repeatedly on the same gel. The Bradford assay (Protein Assay Dye Reagent Concentrate, Bio-Rad 500-0006) was used to determine total protein concentration in purified goldfish VTG and putative purified carp VTG. Samples were diluted in Milli-Q water and 2X treatment buffer (0.125 M Tris, 4% SDS, 20% glycerol, 10% 2-mercaptoethanol, pH 6.8) and heated to 95 °C for 5 min. One μg total protein was added to each well of a pre-cast Tris-glycine mini-gel (4-15% Tris-HCl Ready Gel, Bio-Rad 161-0902 or 161-1104). Following electrophoresis, proteins were electroblotted onto a nitrocellulose membrane (Hybond™ ECL™, Amersham Life Science RPN 2020D) at 70 V for 4 hr in ice-chilled transfer buffer (25 mM Tris, 192 mM glycine, 0.1% SDS, 20% methanol). All proteins on the membranes were stained with Ponceau S (Sigma, St. Louis, Missouri; P-3504) solution to evaluate the completeness of the transfer and the purity of the HPLC fraction thought to contain carp VTG. The membrane was cut into strips and blocked with 3% nonfat dry milk in TBST buffer (TBST: 20 mM Tris, 154

mM sodium chloride, 0.1% Tween-20, pH 7.5; blocking buffer: 3% (w/v) nonfat dry milk in TBST) for 2 hr at room temperature. The strips were probed for positive identification of VTG with two different rabbit anti-goldfish VTG antisera developed previously. One of these antisera was developed at Michigan State University (Nichols, 1997) and demonstrates specificity to cyprinid VTG. The other was generously donated by Dr. Glen Van Der Kraak, Department of Zoology, University of Guelph. The primary antisera were diluted in TBST and incubated with the membranes for 2 hr at room temperature. Following washes with TBST, the membranes were incubated for 1 hr at room temperature with biotinylated donkey anti-rabbit IgG secondary antibody (Amersham Pharmacia Biotech RPN1004) diluted 1:2000 in TBST. After another series of washes, membranes were incubated for 20 min at room temperature with tertiary antibody (streptavidin-peroxidase, Amersham Pharmacia Biotech RPN1231) diluted 1:2000 in TBST. Blots were washed with TBST and developed by incubating them with ECL™ Western blotting reagents (Amersham Pharmacia Biotech RPN2109) according to the instructions provided. Luminescence was detected with a Chemi Doc® 1000/2000 gel documentation system (Bio-Rad Laboratories, Hercules California) controlled by Quantity One® quantitation software, Version 4 (1998, Bio-Rad Laboratories, Hercules, California).

Primary antiserum specificity

SDS-PAGE (as described above) and Western blotting were used to test the lot of polyclonal rabbit anti-goldfish VTG antiserum to be used as the primary antiserum in the VTG ELISA for specificity to carp VTG and nonspecific cross-reactivity with other plasma proteins. For this purpose, the following samples were separated simultaneously on the same gel: HPLC-purified carp VTG, HPLC-purified goldfish VTG, plasma from an uninduced female carp (positive control), plasma from an E2-induced male carp (positive control), plasma from an uninduced male carp, and pooled plasma from uninduced male carp. Biotinylated protein molecular weight markers (broad range, Bio-Rad, St. Louis, Missouri, 161-0319) were used. Electroblotting and immunoblotting were performed as described previously with the following exceptions. Electroblotting was performed with a Trans-Blot® SD semi-dry electrophoretic transfer cell (Bio-Rad, St. Louis, Missouri, 170 3940) using Towbin transfer buffer (25 mM Tris, 192 mM glycine, 20% methanol). For two mini-gels, settings were 15 V, current limit 0.44 A, 30 min. Membranes were blocked overnight at 4 °C.

ELISA protocol

A competitive ELISA for carp VTG was adapted from a method described previously (Nichols et al., 2000). Changes to the original protocol include use of carp VTG rather than goldfish VTG as a standard. Also, certain

incubation steps were lengthened and conducted at lesser temperatures to decrease the possibility of VTG degradation. First, HPLC-purified carp plasma is used to coat the wells of a 96-well plate. Next, primary antiserum and plasma samples are added, and VTG in the samples competes with the pure VTG bound to the plate for binding sites on the primary antiserum. A washing step removes unbound antiserum and sample. A secondary antiserum linked to horseradish peroxidase (HRP) is directed against the primary antiserum and catalyzes a color development reaction that discloses the primary antiserum bound to the purified VTG in the well. Therefore, a lesser concentration of VTG in the sample increases the amount of primary antiserum that binds to the purified VTG on the plate, thus increasing the intensity of the color in the well.

Briefly, the carp VTG ELISA was performed as follows. (A) *Plate coating*. Flat-bottom, high binding, 96-well ELISA plates (Bio-Rad 224-0096) were coated with 45 ng/well purified carp VTG in sodium bicarbonate buffer (50 mM, 5 mg/L gentamicin, pH 9.6). Plates were coated for 24 hr at 4 °C. The contents of the wells were discarded and the wells washed 4 times with 200 µL of wash buffer (TBST: 10 mM Tris, 0.15 M sodium chloride, 0.1% Tween-20, 5 mg/L gentamicin). (B) *Plate saturation*. Unbound surfaces of the wells were blocked by addition of 200 µL/ well blocking buffer, which consists of 2% goat serum (Sigma, St. Louis, Missouri; G-9023) in TBST

(TBST-SG). Plates were incubated at 25 °C for 3 hr. (C) *Addition of samples, standards, and primary antiserum*. Samples were diluted in TBST-SG at a minimum dilution of 1:50 to avoid serum effects. Standards curves were constructed by serial dilution of HPLC-purified carp VTG in TBST-SG. Standards ranged from 5.338 to 2733 ng/mL, resulting in a method detection limit (MDL) of 0.267 μg VTG/mL. After the blocking step, blocking buffer was discarded from the wells and a 50 μL volume of diluted sample or standard was added to each well. Standards and samples were assayed in duplicate on each plate. Primary antiserum (polyclonal rabbit anti-goldfish VTG antiserum) was diluted 1:45,000 in TBST-SG, and 100 μL was added to each well. Wells used to determine nonspecific binding (NSB) received only 150 μL of TBST-SG. Wells used to determine maximum binding received 50 μL TBST-SG and 100 μL diluted primary antiserum. Plates were shaken briefly to mix well contents, then incubated at 25 °C for 12 hr. (D) *Addition of secondary antibody*. Plates were washed to remove unbound antiserum and sample from Step C. The secondary antibody, donkey anti-rabbit IgG linked to HRP (Amersham International NA934), was diluted 1:2000 in TBST-SG, and 150 μL was added to each well. Plates were covered and incubated for 2 hr at 25 °C, then washed 5 times in preparation for the next step. (E) *Color development*. Color development was achieved by addition of HRP substrate solution (OPD solution: 0.5 mg/mL 1,2-phenylene diamine (*o*-phenylene diamine or OPD, Sigma, St. Louis, Missouri;

P-3804) and 0.5 µL/mL 30% hydrogen peroxide in 50 mM ammonium acetate adjusted to pH 5.0 with citric acid (50 mM)). Each well received 150 µL of OPD solution, and plates were incubated on an automatic plate-shaker at room temperature for 30 min in complete darkness. (F) *Color reaction stop and optical density determination*. The color reaction was stopped by addition of 50 µL 5 M sulfuric acid to each well. Plates were shaken for 10 min under conditions previously described. Absorbance was measured with a 96-well plate-reading spectrophotometer (Cayman Autoreader, OEM Version, Cayman Chemical, Ann Arbor, Michigan) controlled by Cayman EIA software (Version 2.0). Optical density (OD) was measured at 492 nm (650 nm reference). (G) *Calculation of sample VTG concentrations*. The standard curve was subjected to a log-logit transformation of VTG concentration in ng/mL versus absorbance. The log-logit regression equation was

$$\text{logit } B/B_0 = m * \log [VTG] + b$$

where:

B = sample absorbance (OD) corrected for NSB,

B_0 = maximum binding in the absence of primary antiserum corrected for NSB,

m = slope of the least squares regression line,

b = y-intercept of the regression line,

logit B/ B_0 = log [(B/B_0) / (1-(B/B_0)].

The equation for the least squares regression line was used to calculate the sample concentration of VTG in ng/mL, corrected for sample dilution.

Quality assurance

Standards and samples were assayed in duplicate. A percent coefficient of variation was calculated on duplicate OD measurements for each sample, and those that exceeded 10% were re-assayed. Because the log-logit transformation does not function well at the extreme ends of the standard curve, samples that produced less than 15% maximum binding were re-assayed at a greater dilution. A single plasma sample was collected and stored in aliquots in the same fashion as the samples and analyzed twice (two duplicate measurements) on each plate. The average of the final VTG concentrations for the two duplicate analyses was calculated for each plate and used to calculate the inter-assay (inter-plate) percent coefficient of variation (inter-assay %CV) (Grotjan et al., 1996). If r^2 for the standard curve regression line was less than 0.95, the plate was re-run. Parallelism of carp plasma VTG sample dilution curves to a standard curve constructed with purified carp VTG was assessed by analysis of covariance (ANCOVA).

Radioimmunoassay of plasma sex steroids

Subsamples of frozen carp blood plasma were shipped on dry ice to the Biotechnology for Evolutionary, Ecological, and Conservation Sciences Program Laboratory at the University of Florida, Gainesville, for analyses of the plasma sex steroids 17β-estradiol (E2), testosterone (T), and 11-ketotestosterone (11-KT) by competitive radioimmunoassay (RIA) (Goodbred et al., 1997). Prior to RIA analysis, duplicate samples were extracted twice with diethyl ether, evaporated to dryness under nitrogen, and reconstituted in RIA buffer (25 mM sodium phosphate monobasic, 0.05 M sodium phosphate dibasic, 0.15 M sodium chloride, 0.25 g/L sodium azide, 1 g/L gelatin, pH 7.5). Standard curves were prepared by analyzing known concentrations of radioinert steroid diluted in RIA buffer. Cross-reactivities of the E2 antiserum with other female sex steroids were 11.2% for estrone; 1.7% for estriol; < 1.0% for 17α-estradiol and androstenedione; and < 0.1% for ethinylestradiol, diethylstilbestrol, and all other steroids examined. Cross-reactivities of the 11-KT antiserum with other male sex steroids were 9.65% for testosterone, 3.7% for α-dihydrotestosterone, < 1.0 percent for androstenedione, and < 0.1% for all other steroids examined.

Statistical analyses

Data were analyzed with the aid of SYSTAT© Version 9 for Windows (SPSS Science, Chicago, 1998). Where data demonstrated normal distributions and

homogeneous variance (homoscedasticity), differences among sites within each sex were examined by one-way analysis of variance (ANOVA) followed by Tukey's HSD post-hoc comparisons of means. When data failed to meet the assumptions of the parametric statistical methods due to heteroscedasticity or pronounced departures from normal distribution, a Kruskal-Wallis (nonparametric) test was approximated by examining the ranks of the data by one-way ANOVA followed by a Tukey-like post-hoc comparison of means (Tukey HSD conducted on the ranked data). Normality was assessed by examining probability plots of the normal distributions and by assessing the results of the SYSTAT© Kolmogorov-Smirnov one-sample test with the Lilliefors option (comparison to the standard normal distribution). Because ANOVA testing is robust with respect to departures from the assumptions of homogeneous variance and normal distribution and because nonparametric testing methods have less power to detect differences among groups (Zar, 1984), only severe departures from normality ($p<0.01$) and homoscedasticity were considered sufficient to necessitate the use of nonparametric testing. When sample sizes were relatively large ($n \geq 20$) within each group (cell) to be compared, the variances were considered to be homogeneous when the ratio of the largest cell variance to the smallest cell variance did not exceed 10:1. In most cases, the data were subjected to parametric and nonparametric analyses, and an attempt was made to resolve any differences in the resulting final interpretation. For

example, extreme values (outliers) might influence the results. In some cases, $\log_{10}$-transformation normalized the data and decreased the variances; this transformation was used where appropriate. The transformed data were re-examined to determine whether the transformation caused the data to meet the parametric assumptions. If the transformed data met the assumptions, they were subjected to the parametric analyses. In general, outliers were not excluded from analyses, particularly analyses of small data sets ($n < 20$), unless this action was justified through best professional judgement. Outliers were not excluded from charts or graphs. Exclusion of outliers from statistical analyses is noted in the discussion of the results. Unless otherwise stated, effects were considered to be statistically significant at the 0.05 level of type I error (α) for all analyses, with the exception of tests used to assess normality of distributions (noted previously).

Multivariate profile analysis (Morrison, 1976) was used to examine patterns of ovarian follicles in different stages or conditions (primary, secondary, or tertiary stages of development). The statistical software package used to aid in the profile analysis was the SAS System for Windows© Release 7.00 (SAS Institute, Cary, North Carolina, 1998). The analysis was conducted on arcsine transformed proportions of follicles (parametric) and also on the ranks of these proportions (nonparametric). The arcsine transformation was

executed to cause proportions to assume a nearly normal distribution (Zar, 1984). Because this transformation does not work well at the extreme ends of the range of possible values, for the proportions X/n (where X= the number of follicles in a particular condition and n= 50 follicles counted), the arcsine transformation was improved by replacing $0/n$ with $1/4n$ and n/n with $1-1/4n$, as suggested by Zar (Zar, 1984).

When plasma samples demonstrated levels of VTG less than the MDL of 0.267 μg VTG/mL, the value of one half of the MDL (0.134 μg VTG/mL) was used for the purposes of statistical analyses. ANCOVA was used to assess the parallelism of carp plasma VTG sample dilution curves to a standard curve constructed with purified carp VTG. ANCOVA was conducted with the aid of the SAS System for Windows© Release 7.00 (SAS Institute, Cary, North Carolina, 1998).

Potential relationships between some endpoints were examined by calculating correlations between them. Pearson product-moment correlation coefficients and Spearman rank correlation coefficients were calculated with the aid of SYSTAT and SAS, respectively. Correlation coefficients were considered to be significant at $p < 0.05$ and highly significant $p < 0.01$.

Box plots were created with SYSTAT (SPSS Science, Chicago, 1998), and information in the box plots is described in the software help manual as follows (paraphrased). The center [horizontal] line marks the median of the sample. The length of each box shows the range within which the central 50% of the values fall, with the box edges (called hinges) at the first and third quartiles. Hspread is comparable to the interquartile range or midrange and is the absolute value of the difference between the values of the two hinges. Fences, which define outside and far outside values, are defined as follows:

Lower inner fence = lower hinge - (1.5 * Hspread)

Upper inner fence = upper hinge + (1.5 * Hspread)

Lower outer fence = lower hinge - (3 * Hspread)

Upper outer fence = upper hinge + (3 * Hspread)

The whiskers show the range of observed values that fall within the inner fences (within 1.5 Hspreads of the hinges). Because the whiskers extend to observed values and the fences need not correspond to observed values, the whiskers do not necessarily extend all the way to the inner fences. Asterisks mark values between the inner and outer fences, and circles mark far outside values, or those beyond the outer fences.

RESULTS

Exposure site water quality characteristics

Water quality parameters measured at each site generally were within tolerance guidelines for optimal health and growth of cyprinid fish (Table 2-2). (Billard, 1999a). The dissolved oxygen reached supersaturation levels at sites LX and LW due to photosynthetic activity of phytoplankton, but the carp did not appear to suffer any obvious adverse effects. Water hardness at all sites reached levels greater than the optimum range for carp, but they can tolerate hardness exceeding 250 mg/L (Billard, 1999a). Hardness was greater at sites LX and LW than at the reference sites. There were consistent water temperature differences among sites (LW > LX > WB > MC), with average site temperatures ranging from 16.0 °C at LW to 13.3 °C at MC. Conductivity measurements were greatest at LW, less at LX, and least at WB and MC, indicating that carp caged at LW were receiving the greatest exposure and those caged at LX were receiving lesser exposure to Las Vegas Wash influent. Conductivity measurements at the reference sites were consistent with conductivity of main body lake water. Unionized ammonia and nitrite are not reported because they were not detected.

Exposure duration and fish survival

Although the goal was to expose carp at the different sites for the same length of time, unpredictable poor weather prevented timely retrieval of the

cages such that the exposure duration varied from 42 to 48 d for pairs of cages (Table 2-3). Exposures began in mid-February and lasted through late March or early April. Four carp died during the exposure: two males caged at WB, one male caged at LX, and one female caged at LX. The sexes of some fish were assigned incorrectly at the beginning of the study, and these fish were placed into cages designated for the opposite sex. The incorrectly sexed fish were not included in subsequent analyses except for calculations of initial and final mean weight per fish in each cage.

Condition

With the exception of male carp caged at MC, the mean weight per fish in each cage was less at the end of the exposure than it was at the beginning (Table 2-4). Condition factors (K) for female carp ranged from 2.1e-1 to 3.6e-1 (Table 2-5). Mean K for female carp ranged among sites from 2.4e-1 to 2.6e-1. For the female fish, parametric and nonparametric analysis yielded similar results, particularly when one outlier was excluded from the parametric analysis. Mean K for female carp caged at LX was significantly less than that for female carp caged at the reference sites WB and MC (Figure 2-2). Mean K for female carp caged at LW was less than that for females caged at MC but not significantly different than mean K for females caged at WB. Mean K for female carp held at HF did not differ significantly from that in females caged at any other site.

Condition factors for male carp caged at all sites ranged from 1.8e-1 to 3.0e-1. Mean K for males ranged among sites from 2.3e-1 to 2.6e-1 (Table 2-5). The male fish demonstrated a pattern of mean K among sites that was similar to the pattern observed in females. Mean K for male carp caged at LW and LX were significantly less than those for males caged at either MC or WB (LW, LX < MC, WB). Mean K for male carp held at HF was significantly different only from that in males caged at WB.

Histology of the hepatopancreas

Hepatopancreas samples taken from 74 male carp and 72 female carp were examined histologically (sample sizes reported in Table 2-6). All but two of the fish examined, a female caged at WB and a male caged at LW, exhibited biliary stasis.

Hepatopancreas vacuolation

The median score for vacuolation of the hepatopancreas was significantly greater in female carp held at HF than in female carp caged at the field sites (Figure 2-3, Table 2-6). No significant differences in median score for vacuolation of the hepatopancreas were observed among females caged at different sites in the lake.

In the male carp, the median score for vacuolation of the hepatopancreas for fish held at HF was significantly greater than that of fish caged at all sites in the lake except WB (Figure 2-3, Table 2-6). No significant differences were observed in vacuolation of the hepatopancreas among male carp caged at the field sites. The median score for vacuolation of the hepatopancreas was significantly different between male and female carp caged at the same site only at WB (males > females).

Gonadosomatic index

The GSI of female carp ranged from 2.2e-2 to 2.2e-1 (Table 2-5). Mean GSI of females ranged among sites from 8.5e-2 (HF) to 1.5e-1. Mean GSI of female carp held at HF was significantly less than mean GSI of females caged at each of the field sites (Figure 2-4). No differences in mean GSI were observed among females caged at the field sites.

GSI of male carp ranged from 1.2e-2 to 9.9e-2 (Table 2-5). Mean GSI of males ranged among sites from 4.6e-2 (HF) to 6.2e-2. No statistically significant differences in mean GSI were observed among male carp caged or held at different sites (Figure 2-4).

Histology of the ovary

Ovary samples from 75 female carp were examined histologically. Sample sizes were as follows: HF (n=12), WB (n= 16), MC (n=15), LW (n= 16), LX (n= 16). One carp caged with the female fish at reference site MC was determined to be intersex upon gross examination; this was confirmed by histological examination of the gonad. The gonad consisted of approximately one-half testicular and one-half ovarian tissue.

Ovarian follicle development

Multivariate profile analysis of arcsine transformed proportions of follicles in each stage of development (primary, secondary, and tertiary) revealed an overall effect of site on the profile of follicle stages. However, carp held at HF were not directly comparable to the carp caged in the field. When carp held at HF were excluded from the analysis, no overall effect of site on follicle development profile was demonstrated among carp caged in the field (Figures 2-5 and 2-6). Female carp caged at all field sites demonstrated a preponderance of primary follicles (average greater than 60%) and more tertiary than secondary follicles. Female carp held at HF had lesser proportions of primary follicles (average 54%) and greater proportions of secondary follicles than carp caged in the field. The ovaries of all female carp subjected to histological examination contained some follicles developed beyond the primary stage.

Ovarian follicle atresia

Among female carp caged in the field, five of the 63 carp subjected to histological examination of the ovary demonstrated atretic follicles, and in each case, only one follicle per 50 follicles examined in each fish was atretic. The carp demonstrating atretic follicles were evenly distributed among the four sites. Among the female carp held at HF, five of the twelve fish examined demonstrated atretic follicles. One carp had six, one had two, and the rest had one atretic follicle in 50 follicles examined per fish.

Histology of the testis

Testis samples from 75 male carp were subjected to histological examination. Sample sizes were as follows: HF (n= 12), WB (n= 15), MC (n= 16), LW (n= 16), and LX (n= 16). No degenerative changes were noted in the testis samples.

Spermatogenic activity

Four male carp were incorrectly presumed to be females at the beginning of the study due to their failure to release milt and lack of any other indication of male gender (nuptial tubercles, male coloration), and they were placed in cages designated for females. Three of these males (one caged at LX, one at MC, and one at WB) were later subjected to histological examination and demonstrated spermatogenically inactive testes. They also did not produce

milt at the end of the exposure period. The fourth male carp sexed incorrectly was caged with the females at LX and did not produce milt at the end of the exposure period. This fish was not subjected to histological examination, so spermatogenic activity of the testis could not be determined. These incorrectly sexed carp were not included in the sample sizes listed above. All of the other male carp used in this study had spermatogenically active testes. Only two male carp that were correctly sexed at the beginning of the study did not produce milt (one at MC and one at LW) at the end of the exposure period, but both demonstrated spermatogenically active testes.

Sertoli cell proliferation

In the male carp that were subjected to histological examination of the testes, median Sertoli cell proliferation scores were not significantly different among males caged at different sites or held at HF. Only two male carp, one at LW and one at LX, exhibited Sertoli cell proliferation, and both demonstrated only mild lesions (score = 1).

Plasma VTG

VTG purification, separation, and identification

During purification by HPLC, carp VTG appeared to elute consistently at 27 min (Figure 2-7). SDS-PAGE and Western blotting performed with two different anti-goldfish VTG antisera on this fraction indicated that it

contained two large molecular weight proteins in bands corresponding to those seen in purified goldfish VTG. One of the two antisera was developed in our laboratory and was previously shown to bind specifically to VTG from goldfish and other cyprinids. The other antiserum was previously developed and characterized at another laboratory. The purified goldfish VTG was obtained from E2-induced goldfish in the same manner as the purified carp VTG. The purity of the carp VTG fraction was verified by lack of other protein bands on nitrocellulose membranes stained with Ponceau S. Following protein transfer from gels to nitrocellulose membranes, gels stained with Coomassie Blue revealed no protein bands other than some faint residual bands where the major protein bands occurred. In the Western blot, faint bands of lower molecular weight proteins in the same lanes as the goldfish and carp VTG bands also cross-reacted with both anti-VTG antisera and were presumed to be minor degradation products of VTG. The molecular weights of the two major protein bands appearing in the HPLC fraction for carp and goldfish were between 116 and 200 kD. This is comparable to previous reports of molecular weights for VTG proteins in carp (two proteins, 150 and 180 kD) (Folmar et al., 1996) and goldfish (132 to 156 kD (Nichols, 1997), 140 to 156 kD (Hori et al., 1979), 140 to 147 kD (de Vlaming et al., 1980)). On the basis of molecular weights, correspondence of bands in the putative purified carp VTG to goldfish VTG bands, and cross-reactivity with two different antisera for cyprinid VTG, the

presence of purified carp VTG in fraction 27 from the HPLC fractionation of carp plasma was confirmed.

Primary antiserum specificity

The rabbit anti-goldfish VTG antiserum lot considered to be a candidate primary antiserum for the VTG ELISA was tested for specificity to carp VTG by SDS-PAGE and Western blotting. The antiserum appeared to bind specifically to VTG in HPLC-purified goldfish VTG, HPLC-purified carp VTG, uninduced female carp plasma, and E2-induced male carp plasma (Figure 2-8). The faint bands of lower molecular weight proteins detected in these lanes appear to be the same minor degradation products of VTG identified in the previous Western blot. The antiserum did not appear to exhibit nonspecific cross-reactivity to proteins in plasma from an uninduced male carp or to proteins in pooled plasma from male carp. When gels were stained with Coomassie Blue following protein transfer, faint residual bands appeared, but these were identical to those appearing on the nitrocellulose membranes after staining with Ponceau S. Increasing the current limit to 0.88 A (for two mini-gels) during electroblotting and/or reducing the concentration of methanol in the transfer buffer might improve the technique.

Quality assurance

Plasma taken from a sexually undeveloped male carp was diluted 1:50 (minimum dilution); 50 µL of this diluted plasma added to NSB wells did not change the measurement of nonspecific binding. The inter-assay %CV for all samples analyzed in the VTG ELISA was 14.2 %. Dilution curves for VTG in female carp plasma and E2-induced male carp plasma were parallel to a standard curve constructed with purified carp VTG diluted in TBST-SG (Figure 2-9).

Measurement of carp plasma VTG

Concentrations of VTG in blood plasma of female carp ranged from 0.024 to 29.7 mg VTG/mL overall (Table 2-7). Median concentrations of plasma VTG in female carp ranged among sites from 3.34 to 10.2 mg/mL. The median concentration of VTG in the plasma of females held at HF was significantly less than the median concentration in females caged at LX (LX > HF) (Figure 2-10). No other differences in median concentration of plasma VTG were detected among female fish caged at different sites.

Among male carp, plasma VTG concentrations ranged from less than the MDL (0.267 µg VTG/mL) to 51.8 µg VTG/mL (Table 2-7). Median plasma VTG concentrations in male carp ranged from 1.04 to 10.7 µg VTG/mL among the sites. The median plasma VTG concentration in male carp caged

at LW was significantly greater than that in males caged or held at the other sites (Figure 2-11). LW also was the only site where all of the caged male carp had detectable concentrations of VTG in their blood plasma. The median concentration of plasma VTG in male carp caged at LW was approximately 3- to 10-fold greater than that in male carp caged or held at the other sites. The minimum concentration observed in a male carp caged at LW was greater than the median concentration in males caged at other sites.

Male and female carp caged at the same site differed significantly in median plasma VTG concentration at each site. Only two male carp had plasma concentrations of VTG that were greater than the least level, 23.7 μg VTG/mL, observed in a female used in this study. These males both were caged at reference sites, one at MC (51.8 μg VTG/mL) and the other at WB (32.1 μg VTG/mL). Concentrations of VTG in the plasma of male and female carp caged at t he same site overlapped only within reference site WB.

Plasma E2

Concentrations of E2 in the plasma of female carp ranged from 92 to 1289 pg E2/mL overall (Table 2-8). Median concentrations ranged from 241 to 653 pg E2/mL among the sites. Among the females, only carp caged at WB

were significantly different, exhibiting a lesser median plasma E2 concentration than female fish caged at the other sites (Figure 2-12).

Concentrations of E2 in the plasma of male carp ranged from 134 to 649 pg E2/mL (Table 2-9). Median concentrations ranged from 316 to 530 pg E2/mL among the sites. The median plasma E2 concentrations in male carp caged at LX and HF were significantly greater than those in male carp caged at both reference sites (Figure 2-12). The median plasma E2 concentration of males caged at LW did not differ significantly from the median concentration at any site other than WB.

Patterns of median plasma E2 concentrations among the various sites were similar between male and female carp. Plasma E2 concentrations in males and females caged at the same sites were significantly different only at MC.

Plasma T

Concentrations of T in the plasma of female carp ranged from 183 to 2660 pg T/mL. Median plasma T concentrations in female carp ranged from 328 to 1303 pg T/mL among the sites. In female carp, the median plasma T concentrations at sites WB and LW were not different from each other but were significantly less than those in carp caged at LX and MC (Figure 2-13). The median plasma T concentration in female carp caged at MC was greater

than that in female carp caged or held at all other sites. The median plasma T concentration in female carp caged at LX was less than that in females caged at MC but not significantly different from that in females caged at HF (WB, LW < LX < MC).

Concentrations of T in the plasma of male carp ranged from 213 to 3462 pg T/mL. Median plasma T concentrations in male carp ranged from 534 to 894 pg T/mL among the sites. Median plasma T concentrations were not significantly different among male fish caged at different sites (Figure 2-13). Median plasma T concentrations were significantly different between male and female carp caged at the same site at HF, LW, and WB, but were not different between males and females caged at MC or LX. Within each site, there was considerable overlap in the range of concentrations of plasma T observed in male and female carp.

Ratio of E2 to T in blood plasma

E2:T ratios in female carp ranged from 0.17 to 3.86. Median and mean E2:T ratios in female carp ranged among sites from 0.35 to 1.52 and from 0.56 to 1.64, respectively (Table 2-8). E2:T data were not normally distributed in all sites and were heteroscedastic. To normalize the data and decrease the variances, E2:T data were $\log_{10}$ transformed. Analysis of the $\log_{10}$-transformed data demonstrated that mean E2:T ratios observed in female

carp caged at LX and LW were not significantly different from those in females caged at both reference sites (Figure 2-14). The mean E 2:T ratio observed in female carp caged at MC was significantly less than that in females caged or held at all other sites. Analysis of the same data by nonparametric methods showed that the median E2:T ratio observed in female carp caged at LW was significantly greater than that in female carp caged at both reference sites. Median E2:T ratio in female carp caged at LX was not significantly different from that observed in females caged at WB or LW. Median E2:11-KT ratio observed in female carp caged at MC was less than that in females caged at all other sites.

Among male carp, E2:T ratios ranged from 0.15 to 1.77. Median and mean E2:T ratios in males ranged among sites from 0.48 to 0.77 and from 0.54 to 0.79, respectively (Table 2-9). Analysis of $\log_{10}$-transformed E2:T data detected a significant difference in mean E2:T ratio only between males caged at MC and LW (Figure 2-14). Mean E2:T ratios observed in male carp caged at LX and LW were not significantly different from those in males caged at both reference sites. Nonparametric analysis detected no significant differences in median E2:T ratios among male carp caged at different sites.

Regardless of the statistical methods used, E2:T ratios were significantly different between male and female carp only within sites HF and LW. Median E2:T ratios of female carp were greater than 0.80 at all sites but MC. Median E2:T ratios were less than 0.80 for males caged at all sites. Mean E2:T ratios for female carp were greater than 0.90 at all sites other than MC and less than 0.80 for males at all sites. There was a substantial overlap in the range of E2:T ratios observed for males and those observed for females caged at all sites. However, at sites HF and LW, this overlap was due primarily to a few extreme values.

Plasma 11-KT

Concentrations of 11-KT in the plasma of female carp ranged from 64 to 3632 pg 11-KT/mL. The greatest concentration observed in the female carp was an extreme case. The next greatest concentration observed was 932 pg 11-KT/mL, and concentrations for all other females were less than 700 pg 11-KT/mL. Both of the greatest concentrations observed in female carp were in those caged at MC. Median plasma 11-KT concentrations in female carp ranged among sites from 239 to 364 pg 11-KT/mL (Table 2-8). Median plasma 11-KT concentrations observed in female carp caged at LX and MC were significantly greater those in females caged at LW or HF (LW, HF < LX, MC) (Figure 2-15). The median plasma 11-KT concentration in female

carp caged at WB was not significantly different from that observed in females at any other site.

Concentrations of 11-KT in the plasma of male carp ranged from 277 to 4364 pg 11-KT/mL. The median plasma 11-KT concentrations observed in male carp were considerably greater than those in females and ranged among sites from 1012 to 1968 pg 11-KT/mL (Table 2-9). The median plasma 11-KT concentrations observed in male carp caged at LX and HF were significantly greater than those in males caged at MC (Figure 2-15). No other significant differences in median plasma 11-KT were observed among males caged at different sites.

Median plasma 11-KT concentrations were significantly different between male and female carp caged within each site, with males exhibiting greater concentrations in each case. The greatest concentration of plasma 11-KT observed in a single female overlapped the least concentration observed in a single male within each site except HF.

Ratio of E2 to 11-KT in blood plasma

Plasma E2:11-KT ratios in female carp ranged from 0.17 to 5.16. Median and mean E2:11-KT ratios for females ranged among sites from 0.76 to 2.63 and from 0.94 to 2.97, respectively (Table 2-8). Plasma E2:11-KT data

were heteroscedastic and not normally distributed. The values were log_{10}-transformed to normalize the data and decrease the variances sufficiently for parametric analysis to be applied. The results of statistical analysis of log_{10}-transformed data were the same as those obtained using untransformed data. Mean E2:11-KT ratio in female carp caged at WB was less than that observed in females caged at all other sites. Mean E2:11-KT ratios were not significantly different among females caged at MC, LX, and LW (Figure 2-16). Mean E2:11-KT ratio was greatest among female carp caged at HF (WB < MC, LX, LW < HF). Mean E2:11-KT ratios observed in females caged at LX and LW did not differ significantly from that in females caged at reference site MC.

Plasma E2:11-KT ratios observed in male carp ranged from 0.14 to 1.13. Median and mean plasma E2:11-KT ratios for males ranged among sites from 0.24 to 0.37 and from 0.29 to 0.44, respectively (Table 2-9). No significant differences in median E2:11-KT ratios were observed among males caged at different sites (Figure 2-16).

Mean plasma E2:11-KT ratios were significantly different between male and female carp within each site. The maximum E2:11-KT ratio found in a single male exceeded the minimum ratio found in a single female within each site except HF. The mean E2:11-KT ratio for females exceeded that observed for

males within each site by more than 3- to 9-fold. Mean E2:11-KT ratios observed at each site were greater than 0.90 for female carp and were less than 0.50 for male carp.

Correlations between biomarkers

Correlations between sex steroid hormones and ratios, other reproductive endpoints (GSI, VTG), and an indicator of health (condition factor K) were calculated in an attempt to identify relationships among the endpoints.

Females

In the female carp, GSI exhibited a significant positive correlation with plasma T, 11-KT, and VTG and a significant negative correlation with plasma E2:T ratio (Table 2-10). However, the linear relationships between these endpoints were weak, as all r^2 values were less than 0.1. Significant but weak ($r^2 < 0.1$) negative correlations were observed between K and the following endpoints: plasma E2, E2:T ratio, and E2:11-KT ratio. Significant but weak ($r^2 \leq 0.11$) positive correlations also were demonstrated between the following pairs of endpoints: E2 and T, E2 and 11-KT, T and 11-KT, and VTG and 11-KT. The variation in E2:T ratio appears to be affected more by its significant negative relationship with T ($r^2 = 0.38$) than by its significant positive relationship with E2 ($r^2 = 0.20$). The variation in E2:11-KT ratio appears to be affected more by its significant positive relationship with E2

($r^2 = 0.46$) than by its significant negative relationship with 11-KT ($r^2 = 0.18$). The strongest relationships between the endpoints examined for female carp existed between E2 and E2:11-KT ratio and between E2:T ratio and T.

Males

Fewer correlations between the endpoints were observed for male carp than for females (Table 2-11). Weak ($r^2 < 0.15$) but significant positive correlations exist between T and E2 and between T and 11-KT in the males. K and E2 exhibited a weak ($r^2 < 0.10$) but significant negative correlation. E2 and 11-KT demonstrated a significant positive correlation ($r^2 = 0.25$). Neither E2:T ratio nor E2:11-KT ratio were significantly correlated with E2; the variation in these ratios was explained primarily by relationship to the androgens ($r^2 = 0.71$ and $r^2 = 0.64$, respectively). The relationships between the androgens and their respective hormone ratios were the strongest correlations observed among the endpoints examined in the males.

DISCUSSION AND CONCLUSIONS

Fish survival and condition

Carp mortalities during the field exposure were few in comparison with those from other fish caging studies conducted in sewage treatment plant effluents (Purdom et al., 1994; Harries et al., 1999) or in rivers receiving them

(Harries et al., 1996; Nichols et al., 1999). This is probably because carp have a greater tolerance for poor water quality conditions than do salmonid species. With one exception, male carp caged at MC, the mean weight per fish in each cage was less than it was at the beginning of the exposure period. This finding might indicate that the fish were stressed simply by being held in cages. Considering the length of exposure (weeks), one would expect the fish to increase in weight rather than decrease. However, as explained previously, the methods used to measure the total weight of fish per cage at the time of cage placement and cage retrieval were different and might have introduced an error. In addition, the method used to determine initial total weight was crude.

The mean condition factors for female carp caged at different sites indicated that those caged at MC exhibited the greatest condition and those caged at LX exhibited the least condition among the sites. Females caged at HF, LW, and WB exhibited similar condition factors. Male carp caged at the reference sites MC and WB exhibited greater condition factors than those caged at LW or LX. Water turbidity was greater at sites LW and LX than it was at MC and WB, and fish at the former two sites might have had more difficulty locating food when it was dropped on the cages from a boat. Although natural food surely was more abundant at LW and LX, the carp were not free to forage and would have competed for any natural food available in the

cages. The greater water temperatures observed at LW and LX relative to the reference sites would have resulted in increased metabolism of food by fish caged at those sites. If food availability was the same among sites, increased metabolism might have led to decreased condition in the carp caged at LW and LX relative to those caged at the reference sites. However, increased temperatures in the optimum range observed at the field sites usually accelerate growth in fish that are provided with adequate food. Differences in water hardness or dissolved oxygen at the LX and LW sites versus the reference sites might have caused the fish at the former two sites to experience more stress than the fish at the reference sites, resulting in decreased condition.

Histology of the hepatopancreas

Almost all of the carp subjected to histological examination of hepatopancreas samples exhibited biliary stasis, a nonspecific lesion characterized by accumulation of bile within hepatocytes and bile canaliculi. Causes of this condition in vertebrates include inappetance, hepatocellular swelling (as occurs with fatty degeneration), bile duct inflammation (cholangitis) or bile duct obstruction due to choleliths or parasites (not observed in these fish), overproduction of bile due to intravascular red blood cell hemolysis, and generalized liver inflammation or toxicosis (not observed in these fish) (S. Fitzgerald, personal communication). In general, jaundice

and biliary stasis are uncommon in fish (S. Fitzgerald, personal communication). However, considering that biliary stasis was present as a mild lesion and was observed in almost all of the fish examined, it is not likely to be related to chemical exposures at the sites.

Gonadal development and histology

Previous studies have demonstrated decreased GSI in male, female, and intersex fish exposed to sewage effluent (Harries et al., 1997; Jobling et al., 1998). Therefore, GSI was examined in carp caged at Lake Mead to determine whether exposure to the Las Vegas Wash intrusion reduced gonadal development. The carp held at HF were sampled earlier than the carp held in the field and are assumed to be representative of the initial condition of carp caged in the field with respect to GSI and plasma VTG concentrations. The lesser mean GSI observed in female carp held at HF relative to those observed in females caged in the field indicated that the female carp caged in the field were undergoing active gonadal growth and development (recrudescence) beyond that present when they were initially placed in the cages. Fish undergoing active gonadal growth and development provide the most useful information regarding the endpoints examined in this study (McMaster et al., 1992). However, the difference in pattern of ovarian follicle development between female fish held at HF and those caged in the field indicated that the difference in GSI might have been

due to disparity in water temperature, light, food availability, or any of a number of other factors. Mean GSI and ovarian follicle development profiles were not significantly different among female carp caged at different sites, indicating that, in general, the female carp were in approximately the same stage of ovarian development at all field exposure sites. The preponderance of primary follicles in the ovaries of female carp caged at all sites also indicated an earlier rather than later stage of gonadal recrudescence. However, because carp are fractional spawners with asynchronous development of ovarian follicles, it is possible that these fish were in the final stages of ovarian recrudescence and maturation or even that some of them already had spawned. Given the average water temperatures measured at the exposure sites, it is unlikely that the fish had spawned. Although carp may spawn at lesser temperatures (> 15 °C) in colder geographic regions (Boon Swee et al., 1966; Shikhshabekov, 1972; Horvath, 1986), the optimum temperature range for spawning and ovulation in carp in temperate regions is approximately 19 to 24 °C (Manning et al., 1984; Aida, 1988; Billard, 1999b). A complete cycle of oogenesis in common carp requires at least 1000 degree-days (or 60 d at 20 °C), and final oocyte maturation generally requires temperatures of greater than 18 to 20 °C (Billard, 1999b). There were no obvious indications that the female carp had ovulated, such as free ova lying in the ovarian lumen (Gupta, 1975) or bloody, flaccid ovarian tissue (Crivelli, 1981). Oocytes in some female carp reached 1.1

mm in diameter, a size indicative of ripe ovaries (Parameswaran et al., 1972; Gupta, 1975; Degani et al., 1996; Billard, 1999b). However, maximum oocyte diameter in carp tends to vary with geographic region and age of fish (Parameswaran et al., 1972; Crivelli, 1981).

Because the female carp demonstrating atresia of ovarian follicles were evenly distributed among the field sites in this study, it is unlikely that atresia was caused by exposure to contaminants entering the lake via the Las Vegas Wash. Incidence and severity of ovarian follicle atresia was greater in female fish held at HF than it was for females caged at any of the field sites. The reason for this difference is unknown, but it appears to be related to some factor associated with conditions at the hatchery. Ovarian follicle atresia has been associated with stress in fish (Tyler et al., 1996). Frequent water changes and cleaning required to maintain the carp in the cement ponds at HF probably caused some stress for the carp, particularly since these fish were reared under conditions that did not habituate them to this type of attention and they frantically avoided anyone approaching the ponds. In contrast, at the two field sites where the caged carp could be observed (MC and WB), the fish did not display any obvious signs of stress and seemed to associate the approach of a boat with food.

Testicular development characterized by GSI was similar among male fish caged or held at all sites. Mean GSI for male carp held at HF was less than that observed in males caged in the field, although the difference was not statistically significant. This indicates that male fish caged in the field probably were undergoing active gonadal growth. On the basis of GSI observed by other researchers in common carp or mirror carp in different states of sexual development, both male and female carp used in this study appeared to be in the mid- to late stages of gonadal recrudescence at the end of the exposure period (Parameswaran et al., 1972; Shikhshabekov, 1972; Gupta, 1975; Crivelli, 1981; Manning et al., 1984; Horvath, 1986; Guha et al., 1987; Tyler et al., 1990).

Four male carp were identified incorrectly and placed into cages designated for females. Three of these carp were subjected to histological examination of the testes, which were determined to be spermatogenically inactive. Because some of the fish used in this study were only two years old, it is possible that these four fish simply had not reached sexual maturity prior to caging. Other potential causes of this condition include seasonal inactivity, secondary to generalized debilitation (emaciation, severe parasitism, septicemia, aging- not observed here), an endocrine imbalance, and toxicosis. However, all of the other male carp examined were actively spermatogenic and most were spermiating (producing milt). Because

spermatogenically inactive testes were noted only in three carp, all caged at different sites, and all mistaken for females at the beginning of the study, it is extremely unlikely that toxicity was the cause of the condition. Only two male carp that were sexed correctly at the beginning of the exposure period were not spermiating (producing milt) at the end of the exposure period, but both demonstrated spermatogenically active testes.

Only two male carp, one caged at LX and one at LW, exhibited Sertoli cell proliferation. Both of these males had only mild lesions, but the one caged at LW was one of the two male carp in this study that failed to produce milt. Although it is possible that the Sertoli cell proliferation was related to contaminants in the Las Vegas Wash, the low incidence and mild nature of the lesions do not provide strong evidence toward that conclusion. Exposure of male fathead minnows (*Pimephales promelas*) to 2 nM (5.4 ng/L) E2 for 14 d (or 19 d) or to 1.6 to 3.4 μg/L nonylphenol (NP) for 42 d caused severe or moderate Sertoli cell proliferation in the testes, respectively (Miles-Richardson et al., 1999b). In 1997, concentrations of E2 and ethinylestradiol (EE2) were measured at all of the sites where these carp were caged in 1999 (Table 2-12). The concentration of E2 measured in water at LW in 1997 was 2.67 ng/L, a concentration approaching that required to cause Sertoli cell proliferation in male fathead minnows. Other estrogenic chemicals, including EE2 (a potent estrogenic component of oral

birth control medications) and NP (an estrogenic degradation product of non-ionic surfactants) also were present in these water samples at pg/L or ng/L concentrations, respectively.

Intersex carp

One carp caged with the female carp at MC demonstrated an intersex gonad. Estrogenic contaminants associated with sewage treatment plant effluents have been implicated as a potential cause of an unusual incidence of an intersex condition observed in wild roach (*Rutilus rutilus*) in rivers in the United Kingdom (Jobling et al., 1998). Exposure to certain alkylphenolic compounds via water can induce the formation of ovo-testis in male common carp (Gimeno et al., 1996) and Japanese medaka (Gray et al., 1997) exposed to these chemicals during critical periods of sexual differentiation. Ovo-testis also can be induced in female carp by oral administration of androgenic steroids such as methyltestosterone applied to feed (Nagy et al., 1981). However, it is unlikely that the single incidence of intersex gonad observed in a carp caged in Lake Mead resulted from exposure to chemicals in the environment during the course of the study. The fish used in this study were adults that were sexually differentiated prior to the commencement of the study, and thus past the most sensitive, sexually labile period of development. It is possible for estrogenic steroids to induce ovo-testis in adult fish. Exposure of sexually mature male common carp for

2 or 3 months to E2 at 1 μg/L caused formation of ovo-testes in previously regressed testes of some exposed fish (Gimeno et al., 1998). However, exposure to relatively great concentrations of alkylphenols or steroids seems to be required to produce this effect in adult fish. Such great concentrations of hormonally active agents were not likely to have been encountered at the reference site where the intersex carp was discovered. Also, in the study conducted by Gimeno et al. discussed previously (Gimeno et al., 1998), regression of the testes appeared to be a prerequisite for formation of ovo-testis. Testicular regression was not observed in carp used in this study. Therefore, it is most likely that the carp already had an intersex gonad prior to being caged in the lake. This might indicate that the carp used in this study already had been exposed to a hormonally active agent prior to purchase. For example, constituents of some fish food formulations have been shown to have estrogenic effects in sturgeon (Pelissero et al., 1991). However, carp used in this study were raised in earthen ponds on natural food and were not treated with hormones to influence sexual phenotype or growth. Occasionally, intersexuality appears to occur spontaneously under natural conditions in cyprinid fish (Atz, 1964).

Plasma VTG

Plasma VTG levels in the female carp do not appear to demonstrate an effect of field site that could be deemed related to chemical exposure. However,

the female fish were undergoing vitellogenesis, and any potential effects might have been masked by the naturally elevated VTG levels. It is interesting that only female carp caged at LX demonstrated a median plasma VTG concentration significantly greater than that observed in female carp held at HF. The carp held at HF were not directly comparable to those caged in the field, but they might represent the plasma VTG concentrations that were present in the female carp when they were first received at HF prior to cage deployment. In the female carp caged at the field sites, the pattern of plasma E2 concentrations and the pattern of plasma VTG concentrations among sites are similar. However, differences among sites for both endpoints were not statistically significant with the exception of a significantly lesser mean plasma E2 concentration in female carp caged at WB relative to females caged at the other sites.

Male carp caged at LW demonstrated elevated concentrations of VTG in their blood plasma relative to the male carp caged or held at the other sites. This elevation in plasma VTG appears to be accompanied by a concomitant, but not statistically significant, increase in median plasma E2 concentration relative to those observed in males at both reference sites; this might account for the difference in plasma VTG. However, male carp caged at LX or held at HF had the greatest median concentrations of plasma E2, similar to that observed in the male carp caged at LW, but did not exhibit an

increase in plasma VTG. This finding suggests that male carp caged at LW were exposed to an exogenous estrogenic substance. Alternatively, this could mean that the levels of plasma E2 in male carp at HF and LX increased very recently relative to a longer-term elevation in males caged at LW, such that there was not sufficient time for a VTG response to be induced at the former two locations. The increase in plasma VTG observed in the male carp caged at LW is not dramatic compared to the 500- to 100,000-fold induction observed in male rainbow trout exposed to sewage effluent in the UK (Purdom et al., 1994). However, when juvenile common carp and rainbow trout were exposed to EE2 or estrogenic sewage effluents in parallel studies, the carp demonstrated a far lesser VTG response than the trout, although the difference in response might have been temperature-dependent (Purdom et al., 1994). The concentrations of plasma VTG observed in the male carp held at the reference sites were surprisingly great (low μg/mL) compared to those observed in sexually mature male carp used in other studies conducted in Europe (low ng/mL or less) (Tyler et al., 1990; Gimeno et al., 1998). Concentrations of VTG observed in the plasma of female carp caged at the reference sites also were greater than those reported by researchers in the UK for unexposed female carp (maximum 1 mg/mL) (Tyler et al., 1990). However, the plasma VTG concentrations observed in male and female carp in our study are comparable to those reported for carp by other researchers in the United States (US) (Bevans et al., 1996; Folmar et al., 1996;

Goodbred et al., 1997). The induction observed in male carp caged at LW in this study is not as dramatic as that reported for wild male carp caught in the Las Vegas Bay and Las Vegas Wash by USGS researchers in May 1995 (Bevans et al., 1996). Bevans et al. (1996) reported low mg/mL concentrations of plasma VTG in male carp exposed to the influent of the Las Vegas Wash, as opposed to concentrations less than 1 mg VTG/mL in male carp caught at their reference site. However, the wild fish might have received a much longer exposure to contaminants in the water or to contaminants biomagnified in the food chain, perhaps during critical periods of sexual development. Interestingly, in the same USGS study, female carp caught in Las Vegas Bay demonstrated greater plasma VTG concentrations than female carp caught in the Las Vegas Wash (Bevans et al., 1996). This parallels the pattern of plasma VTG concentrations observed in female carp in our study. In the present study, only two male fish demonstrated plasma VTG levels greater than the minimum concentration found in a female, and both males were caged at the reference sites MC (51.8 μg/mL) and WB (32.13 μg/mL). In contrast, concentrations of plasma VTG observed in wild male carp collected from LX and LW in the USGS study demonstrated a substantial overlap with concentrations observed in females (Bevans et al., 1996), perhaps signifying a more severe effect on male carp than was observed in our study. Likewise, other researchers have reported concentrations of plasma VTG as great as 10 mg/mL in wild male carp

caught in a sewage effluent canal in Minnesota (Folmar et al., 1996). In agreement with our findings, others also have observed some unexpectedly great concentrations of plasma VTG in control immature male carp used in their studies (Purdom et al., 1994).

Plasma sex steroid hormone concentrations

With regard to plasma sex steroid concentrations, we were concerned primarily with determining whether the influent of the Las Vegas Wash at LW or LX significantly affected estrogen to androgen (E:A) ratios (E2:T ratio or E2:11-KT ratio) in the caged carp in a manner that could not be explained by site physical characteristics. Plasma E2:T and E2:11-KT ratios were similar among male carp caged or held at the various sites. Average water temperatures at the exposure sites exhibited a pattern (MC < WB < LX < LW) that paralleled the pattern of median E2:T ratios observed among females caged at these sites (MC < WB < LX < LW), although the differences in median E2:T ratios were not necessarily statistically significant. Because T is the precursor for synthesis of E2 in the teleost ovary (McMaster et al., 1995), it appears that conversion of T to E2 might have increased with water temperature. T also is the precursor for 11-KT production, and the pattern of median plasma 11-KT concentrations among females caged at the field sites was similar to the pattern observed in median plasma T.

Mean plasma E2:11-KT ratios in female carp were similar among all of the field sites but WB, where females demonstrated a lower ratio than females caged at the other sites, apparently because median plasma E2 concentration was less for females caged at that site. The reason for this is not clear, but median plasma T and E2 concentrations for females caged at WB also were on the lower end of the range of median concentrations among sites. Female carp caged at LW and LX exhibited E2:11-KT ratios that were not significantly different from that in females caged at MC. In short, E:A ratios in male fish do not appear to have been affected by any site-related factors, and differences in E:A ratios observed among female carp caged at the various field sites might be explained by site temperature differences. In the male carp, variation in both E:A ratios was explained primarily by their associations with the androgen. This finding is in accordance with the predominance of the androgens T and 11-KT among the steroids in male carp (Barry et al., 1990). In the female carp, variation in E2:11-KT was affected more by its association with E2 than with 11-KT. E2 is one of the most important sex steroids in female fish (McMaster et al., 1995), whereas 11-KT is more important in male fish, including carp (Barry et al., 1990). Variation in E2:T in female carp was affected more by its relationship with T than with E2. Although T is an androgen and an important sex steroid in male fish, it is also important in female fish (McMaster et al., 1995). Because T serves as a precursor for E2 synthesis

in female fish (McMaster et al., 1995), it is not surprising that T concentrations strongly influence the variation in E2:T ratio in female carp.

Previous studies conducted with male carp captured from areas influenced by sewage effluent have reported decreased serum T (Folmar et al., 1996) or decreased plasma 11-KT (Bevans et al., 1996) along with increased plasma VTG. No decreases in plasma androgen concentrations were observed in male carp exposed to Las Vegas Wash influent in the present study. In the 1995 USGS study on Lake Mead carp, increased plasma 11-KT in female carp caught at LW and decreased plasma E2 in male carp caught in LX were reported. No such effects were observed in the caged carp used in the present study.

Steroid hormone concentrations overall were rather low but similar to concentrations reported previously for adult male and female carp held at 18 to 20 °C (Yano et al., 1986) or for wild-caught carp undergoing gonadal recrudescence (Goodbred et al., 1997). The low steroid hormone concentrations observed in carp caged at the field sites might be representative of typical inter-spawning concentrations or might indicate that the fish at all sites were experiencing stress that prevented their sex steroid concentrations from increasing. Stress is known to cause significant reductions in circulating sex steroid concentrations in some species of fish

(McMaster et al., 1992), possibly including carp (Santos et al., 1986). The failure of the carp to gain weight over the course of the study lends support to the hypothesis that stress prevented sex steroid cycling. However, the similarity of circulating plasma sex steroid concentrations observed in the caged carp to concentrations reported previously in unstressed fish indicated that these are normal levels for carp between spawning periods.

In conclusion, the evidence presented here suggested that male carp caged in the Las Vegas Wash were exposed to an exogenous estrogenic substance, as evidenced by a 3- to 10-fold increase in median plasma VTG concentration in male carp caged in the Las Vegas Wash relative to median concentrations observed in male carp caged at other sites. However, because the water temperature was greater at the Las Vegas Wash site than at the remaining sites, a temperature-related effect cannot be ruled out. No significant impacts of Las Vegas Wash influent on GSI, ovarian follicle development, or steroid hormone profiles were demonstrated that could not also possibly be explained by site physical characteristics. Because no causal link has been established between low-level VTG induction and impacts on reproduction or survival in fish, it is not known whether this represents an effect likely to cause deleterious impacts at the population level. The next logical step at this point is to attempt to identify the chemical constituent(s) of the Las Vegas Wash that might result in VTG

induction in male carp and to determine whether this contaminant or mixture of contaminants might produce effects in fish that would result in a population-level impact or negative effects in the endangered razorback sucker.

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Table 2-1. Descriptions of different ovarian follicle types identified in common carp ovaries during histological examination. Taken from (Miles-Richardson et al., 1999).

Follicle Type	Description
Primary	abundant basophilic cytoplasm (deep blue color), large pale central nucleus, no yolk vesicles
Secondary	eosinophilic yolk vesicles appear in vacuolated cytoplasm, granulosa cells surround central oocyte
Tertiary	largest follicle type, numerous eosinophilic yolk globules fill cytoplasm
Atretic	degenerative follicle with collapsed, irregular contour; macrophages invading follicle

Table 2-2. Water quality parameters measured at caged carp exposure sites in Lake Mead, Nevada. Temp. = temperature; DO = dissolved oxygen; Cond. = conductivity; Turb. = turbidity, in national turbidity units. [a] Measured as $CaCO_3$. [b] Total ammonia.

Site: LX

Date	Temp. (° C)	pH	DO (mg/L)	Cond. (μS)	Turb. (NTU)	Alkalinity (mg/L)	Hardness[a] (mg/L)	Ammonia[b] (mg/L)
03.05	15.8	8.08	9.57	1464	3.7			
03.10						105	306	0.3
03.18	15.3	8.50	12.58	1197	1.4	108	360	0.0
03.26	16.9	8.61	12.83	1088	0.8			
Average	16.0	8.40	11.66	1250	2.0	107	333	0.2

Site: MC

Date	Temp. (° C)	pH	DO (mg/L)	Cond. (μS)	Turb. (NTU)	Alkalinity (mg/L)	Hardness[a] (mg/L)	Ammonia[b] (mg/L)
03.05	12.6	8.05	9.66	893	4.6			
03.10						110	272	0.3
03.18	13.2	8.14	9.58	922	0.2	107	268	0.0
03.26	14.2	8.23	10.13	894	0.0			
Average	13.3	8.14	9.79	903	1.6	109	270	0.2

Table 2-2. (Continued). Water quality parameters measured at caged carp exposure sites in Lake Mead, Nevada. Temp. = temperature; DO = dissolved oxygen; Cond. = conductivity; Turb. = turbidity, in national turbidity units. [a] Measured as $CaCO_3$.
[b] Total ammonia.

Site: WB

Date	Temp. (° C)	pH	DO (mg/L)	Cond. (μS)	Turb. (NTU)	Alkalinity (mg/L)	Hardness[a] (mg/L)	Ammonia[b] (mg/L)
03.10						110	270	0.2
03.19	14.0	8.11	9.57	895	0.4	110	134	0.0
03.26	14.9	8.00	9.83	883	0.4			
Average	14.5	8.06	9.70	889	0.4	110	202	0.1

Site: LW

Date	Temp. (° C)	pH	DO (mg/L)	Cond. (μS)	Turb. (NTU)	Alkalinity (mg/L)	Hardness[a] (mg/L)	Ammonia[b] (mg/L)
03.05	16.2	8.10	9.58	1623	2.8			
03.10						108	336	0.2
03.18	16.4	8.50	13.31	1423	3.4	107	360	0.0
03.26	18.7	8.53	13.17	1636	4.1			
Average	17.1	8.38	12.02	1561	3.4	108	348	0.1

Table 2-3. Fish cage placement and retrieval dates, exposure duration, mortalities, and other general data.

Cage (site/sex)	Placement Date	Retrieval Date	Exposure Duration (d)[a]	Initial Number	Final Number	Mortalities	Number Sexed Incorrectly[c]
LW male	02.17	04.05	47	29[b]	29	0	0
LW female	02.17	04.05	47	30	30	0	0
WB male	02.15	03.29	42	30	28	2	0
WB female	02.15	03.29	42	30	30	0	1
MC male	02.16	04.01	44	28[b]	28	0	0
MC female	02.16	04.01	44	30	30	0	2
LX male	02.18	04.07	48	30	29	1	0
LX female	02.18	04.07	48	30	29	1	2

[a] including day of placement, but not day of retrieval

[b] deaths occurred during transport or placement in cages

[c] number of fish sexed incorrectly and placed into a cage designated for the opposite sex

NA = not applicable

Table 2-4. Weights of carp at initiation and completion of the field exposure period.

CAGE (site/sex)	INITIAL		FINAL	
	Total Wt. (kg)	Mean Wt. per Fish (g)[a]	Total Wt. (kg)	Mean Wt. per Fish (g)[b]
HF male	NA	NA	6.23	160
HF female	NA	NA	3.32	107
LW male	4.90	163	4.62	159
LW female	3.68	123	3.42	114
WB male	5.20	173	4.78	171
WB female	4.08	136	3.72	124
MC male	5.52	184	5.30	189
MC female	4.22	141	3.69	123
LX male	5.62	187	5.16	178
LX female	4.60	153	3.35	115

[a] initial total weight (kg) divided by 30 fish (number initially weighed); converted to grams

[b] final total weight (kg) divided by number of fish when cage was retrieved; converted to grams

NA = not applicable

Table 2-5. Length, weight, gonadosomatic index (GSI), and condition factor (K) data for adult male and female common carp caged at Lake Mead. Means are reported as mean ± 1 SEM.

Site	MALES			FEMALES		
	n	mean	range	n	mean	range
HF						
Length (cm)	12	19.4 ± 0.5	16.7 - 23.3	12	16.8 ± 0.6	14.3 - 21.3
Weight (g)	12	177 ± 14	113 - 296	12	120 ± 12	74 - 223
GSI	12	4.6e-2 ± 3.6e-3	2.5e-2 - 6.2e-2	12	8.5e-2 ± 1.2e-2	2.2e-2 - 1.5e-1
K	12	2.4e-1 ± 2.9e-3	2.2e-1 - 2.5e-1	12	2.5e-1 ± 5.1e-3	2.1e-1 - 2.8e-1
WB						
Length (cm)	28	18.7 ± 0.3	14.8 - 22.1	29	16.9 ± 0.3	14.2 - 20.0
Weight (g)	28	171 ± 9	89 - 269	29	125 ± 7	70 - 202
GSI	15	6.2e-2 ± 5.1e-3	1.2e-2 - 8.6e-2	15	1.3e-1 ± 9.7e-3	3.0e-2 - 1.8e-1
K	28	2.6e-1 ± 3.2e-3	2.3e-1 - 3.0e-1	29	2.6e-1 ± 3.2e-3	2.3e-1 - 2.9e-1
MC						
Length (cm)	28	19.6 ± 0.4	16.2 - 22.8	28	16.7 ± 0.3	13.0 - 20.4
Weight (g)	28	189 ± 11	110 - 288	28	126 ± 7	55 - 218
GSI	16	5.6e-2 ± 4.7e-3	2.5e-2 - 9.9e-2	15	1.5e-1 ± 1.1e-2	6.0e-2 - 2.2e-1
K	28	2.4e-1 ± 3.9e-3	1.8e-1 - 2.8e-1	28	2.6e-1 ± 4.9e-3	2.3e-1 - 3.6e-1

Table 2-5. (Continued). Length, weight, gonadosomatic index (GSI), and condition factor (K) data for adult male and female common carp caged at Lake Mead. Means are reported as mean ± 1 SEM.

Site	MALES			FEMALES		
	n	mean	range	n	mean	range
LW						
Length (cm)	29	18.9 ± 0.4	16.3 - 23.5	30	16.6 ± 0.2	14.1 - 19.1
Weight (g)	29	159 ± 9	102 - 284	30	114 ± 5	69 - 182
GSI	16	5.0e-2 ± 3.8e-3	1.6e-2 - 6.9e-2	16	1.3e-1 ± 1.3e-2	6.5e-2 - 2.1e-1
K	29	2.3e-1 ± 3.3e-3	1.9e-1 - 2.8e-1	30	2.5e-1 ± 3.7e-3	2.2e-1 - 3.3e-1
LX						
Length (cm)	29	19.6 ± 0.4	16.4 - 23.8	27	16.6 ± 0.4	14.0 - 20.9
Weight (g)	29	178 ± 11	109 - 318	27	114 ± 8	67 - 218
GSI	16	5.4e-2 ± 5.0e-3	1.8e-2 - 9.6e-2	16	1.4e-1 ± 8.2e-3	8.1e-2 - 1.9e-1
K	29	2.3e-1 ± 3.3e-3	1.9e-1 - 2.6e-1	27	2.4e-1 ± 2.5e-3	2.2e-1 - 2.8e-1

Table 2-6. Scores for hepatocellular vacuolation of the hepatopancreas for male and female carp, reported as the site median score with minimum and maximum values in parentheses. Vacuolation was scored on a scale as follows: 0 = no vacuolation, 1 = mild vacuolation with small vacuoles spread throughout the cytoplasm, 2 = moderate vacuolation with larger coalescing vacuoles appearing as large clear zones in many hepatocytes, 3 = severe vacuolation where all or most of the cytoplasm has lost its normal pink coloration due to confluent, large, clear vacuoles.

	MALES			FEMALES		
SITE	n	Score	Range	n	Score	Range
HF	12	3	(1 - 3)	11	3	(1 - 3)
WB	15	2	(1 - 3)	16	1	(0 - 2)
MC	16	2	(1 - 2)	13	2	(1 - 2)
LW	15	2	(1 - 2)	16	1	(0 - 2)
LX	16	1.5	(1 - 3)	16	1.5	(0 - 2)

Table 2-7. Concentrations of vitellogenin (VTG) in blood plasma of adult male (μg/mL) and female (mg/mL) carp. Site medians are reported with minimum and maximum values in parentheses. Note that male plasma VTG concentrations are reported in micrograms per milliliter and female plasma VTG concentrations in milligrams per milliliter. ND = not detected (< 0.267 μg/mL).

	MALES		FEMALES	
SITE	n	VTG (μg/mL)	n	VTG (mg/mL)
HF	12	3.16 (ND - 5.47)	12	3.34 (0.176 - 14.2)
WB	28	2.97 (ND - 32.1)	29	8.38 (0.024 - 15.5)
MC	28	2.61 (ND - 51.8)	28	9.20 (0.070 - 19.9)
LW	29	10.7 (5.39 - 20.8)	29	7.99 (0.062 - 19.8)
LX	29	1.04 (ND - 16.3)	27	10.2 (1.52 - 29.7)

Table 2-8. Concentrations of E2, T, and 11-KT, and ratios of E2:T and E2:11-KT in blood plasma of adult female carp. Site medians are reported with minimum and maximum values in parentheses. E2 = 17β-estradiol, T = testosterone, 11-KT = 11-ketotestosterone.

		FEMALES				
SITE	n	E2 (pg/mL)	T (pg/mL)	E2:T	11-KT (pg/mL)	E2:11-KT
HF	12	653 (195 - 1289)	430 (248 - 777)	1.52 (0.79 - 3.86)	239 (64 - 398)	2.63 (1.97 - 5.16)
WB	28	241 (92 - 903)	328 (183 - 715)	0.82 (0.27 - 3.03)	305 (175 - 611)	0.76 (0.31 - 2.65)
MC	28	487 (301 - 760)	1303 (348 - 2660)	0.35 (0.17 - 1.41)	364 (118 - 3632)	1.29 (0.17 - 2.74)
LW	30	462 (269 - 683)	355 (202 - 607)	1.19 (0.70 - 2.44)	272 (225 - 516)	1.56 (0.58 - 2.62)
LX	27	553 (273 - 1001)	549 (284 - 1211)	0.86 (0.30 - 2.53)	352 (230 - 667)	1.53 (0.58 - 2.64)

Table 2-9. Concentrations of E2, T, and 11-KT, and ratios of E2:T and E2:11-KT in blood plasma of adult male carp. Site medians are reported with minimum and maximum values in parentheses. E2 = 17β-estradiol, T = testosterone, 11-KT = 11-ketotestosterone.

		MALES				
SITE	n	E2 (pg/mL)	T (pg/mL)	E2:T	11-KT (pg/mL)	E2:11-KT
HF	12	530 (431 - 649)	894 (266 - 3462)	0.58 (0.15 - 1.73)	1968 (568 - 4364)	0.24 (0.14 - 1.13)
WB	28	316 (134 - 451)	534 (213 - 837)	0.59 (0.29 - 1.37)	1012 (581 -2125)	0.29 (0.15 - 0.55)
MC	28	336 (226 - 596)	750 (290 - 2916)	0.48 (0.15 - 1.06)	1088 (277 - 2593)	0.36 (0.17 - 0.85)
LW	29	435 (239 - 570)	639 (257 - 1509)	0.77 (0.34 - 1.77)	1164 (412 - 2517)	0.37 (0.17 - 1.07)
LX	29	465 (249 - 582)	545 (341 - 1546)	0.66 (0.35 - 1.31)	1549 (382 - 2691)	0.29 (0.18 - 0.94)

Table 2-10. Spearman rank correlation coefficents (r) between plasma sex steroid concentrations and ratios, gonadosomatic index (GSI), condition factor (K), and plasma vitellogenin (VTG) in adult female carp. E2 = 17β-estradiol, T = testosterone, 11-KT = 11-ketotestosterone.

Endpoint	GSI	K	E2	T	11-KT	E2:T	E2:11-KT	VTG
GSI	1.000							
K	0.159	1.000						
E2	-0.015	-0.308**	1.000					
T	0.260*	0.000	0.314**	1.000				
11-KT	0.242*	-0.083	0.272**	0.326**	1.000			
E2:T	-0.272*	-0.240**	0.443**	-0.618**	-0.169	1.000		
E2:11-KT	-0.209	-0.201*	0.681**	0.048	-0.421**	0.510**	1.000	
VTG	0.251*	-0.020	0.101	0.121	0.181*	-0.047	-0.042	1.000

* $0.01 <$ or $=$ $p < 0.05$ for correlation coefficient
** $p < 0.01$ for correlation coefficient

Table 2-11. Correlation coefficents (r) between plasma sex steroid concentrations and ratios, gonadosomatic index (GSI), condition factor (K), and plasma vitellogenin (VTG) in adult male carp. Correlation coefficients are Spearman rank correlation coefficients unless otherwise noted. E2 = 17β-estradiol, T = testosterone, 11-KT = 11-ketotestosterone.

Endpoint	GSI	K	E2	T	11-KT	E2:T	E2:11-KT	VTG
GSI	1.000							
K	0.035 [a]	1.000						
E2	-0.159	-0.261**	1.000					
T	-0.188	-0.101	0.360**	1.000				
11-KT	-0.035	-0.058	0.498**	0.191*	1.000			
E2:T	0.041	-0.014	0.132	-0.843**	0.029	1.000		
E2:11-KT	-0.080	-0.104	0.055	0.025	-0.797**	0.076	1.000	
VTG	-0.058	-0.038	0.041	-0.031	-0.035	0.075	0.049	1.000

[a] Pearson product-moment correlation coefficient

* $0.01 \leq p < 0.05$ for correlation coefficient

** $p < 0.01$ for correlation coefficient

Table 2-12. Concentrations of selected estrogenic contaminants measured in water at various sites where carp were later exposed in cages in Lake Mead, Nevada. Samples were collected April 30, 1997, and/or September 5, 1997. Where ranges are reported, samples were collected on both dates. Taken from (Snyder et al., 2000).

Contaminant	SITE			
	Las Vegas Wash (LW)	Las Vegas Bay (LX)	Moon Cove (MC)	Water Barge Cove (WB)
E2 (ng/L)	2.67	0.188 - 2.21	ND	ND
EE2 (ng/L)	0.480	0.253 - 0.520	ND	ND
NP (ng/L)	1140	160 - 750	ND	ND
OP (ng/L)	43	ND - 27	ND	ND

E2 = 17β-estradiol, EE2 = ethinylestradiol, NP = nonylphenol, OP = octylphenol, MDL = method detection limit. E2 and EE2 were measured by radioimmunoassay. NP and OP were measured by HPLC-fluorescence. MDL for E2 = 107 pg/L, MDL for EE2 = 53 pg/L, MDL for NP = 5.0 ng/L, MDL for OP = 1.0 ng/L.

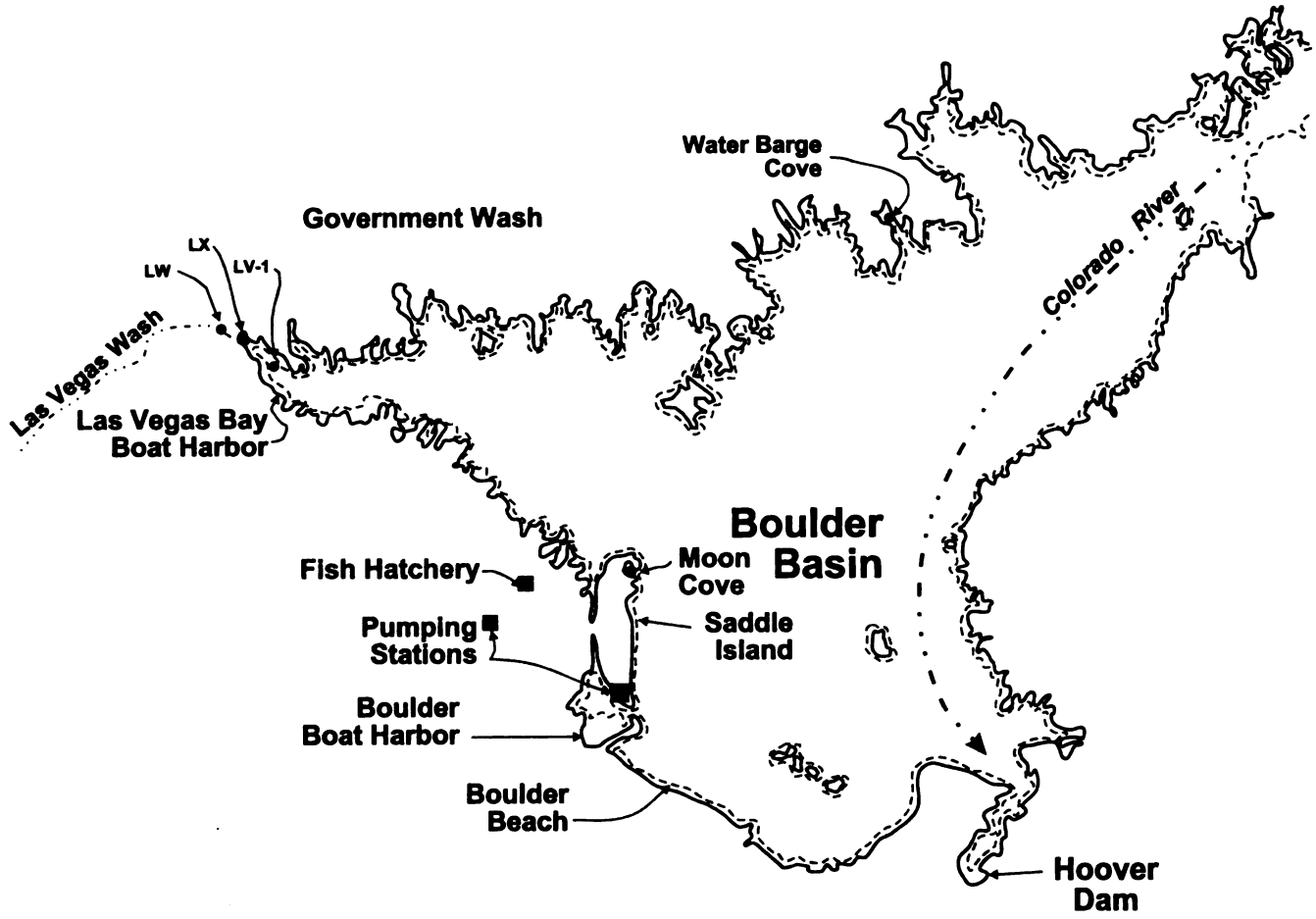

Figure 2-1. Map of the Boulder Basin in Lake Mead, Nevada. Sites of interest in this study are the Fish Hatchery (HF), Moon Cove (MC), Water Barge Cove (WB), Las Vegas Wash (LW), and Las Vegas Bay (LX).

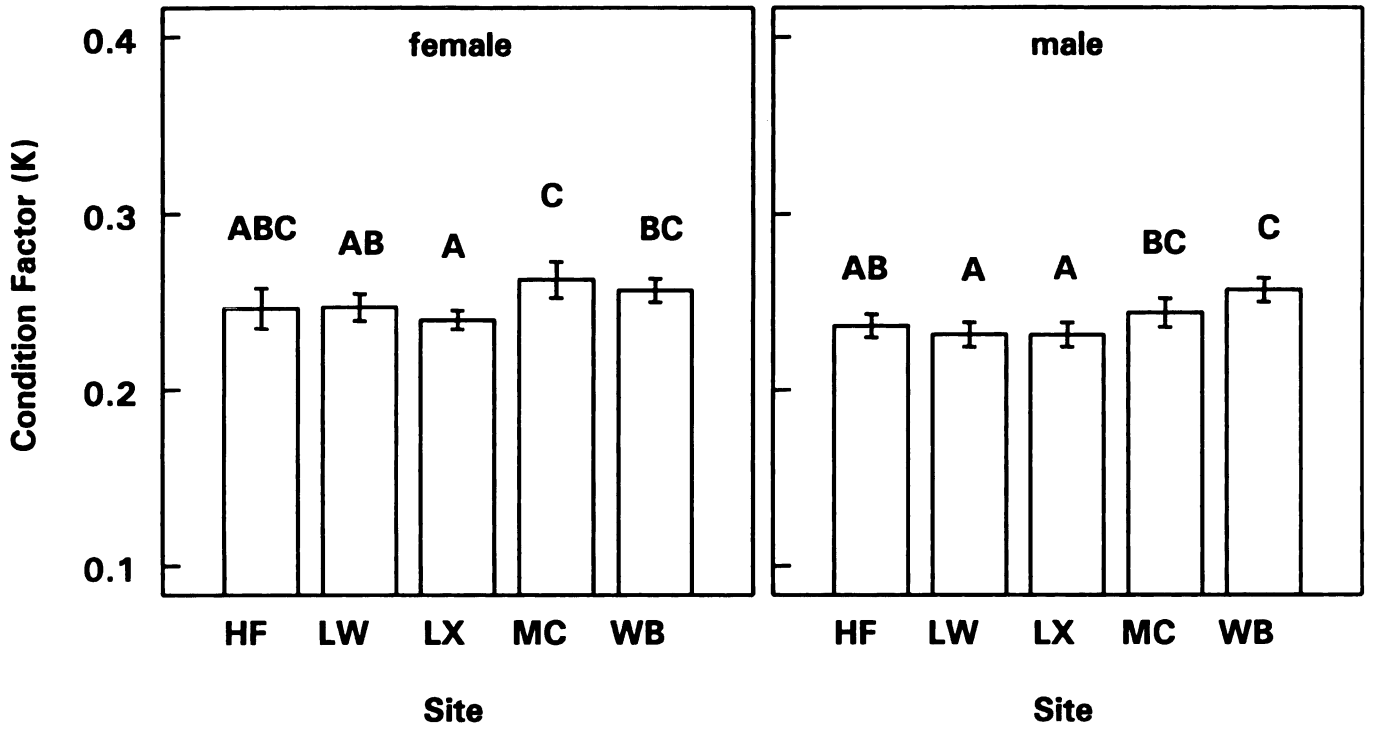

Figure 2-2. Condition factor (K), calculated as $K = W/L^3 \times 10{,}000$ where W = weight in g and L = standard length in mm. Bar heights represent means, and error bars are 2 SEM. Letters above the bars represent Tukey groupings within each sex.

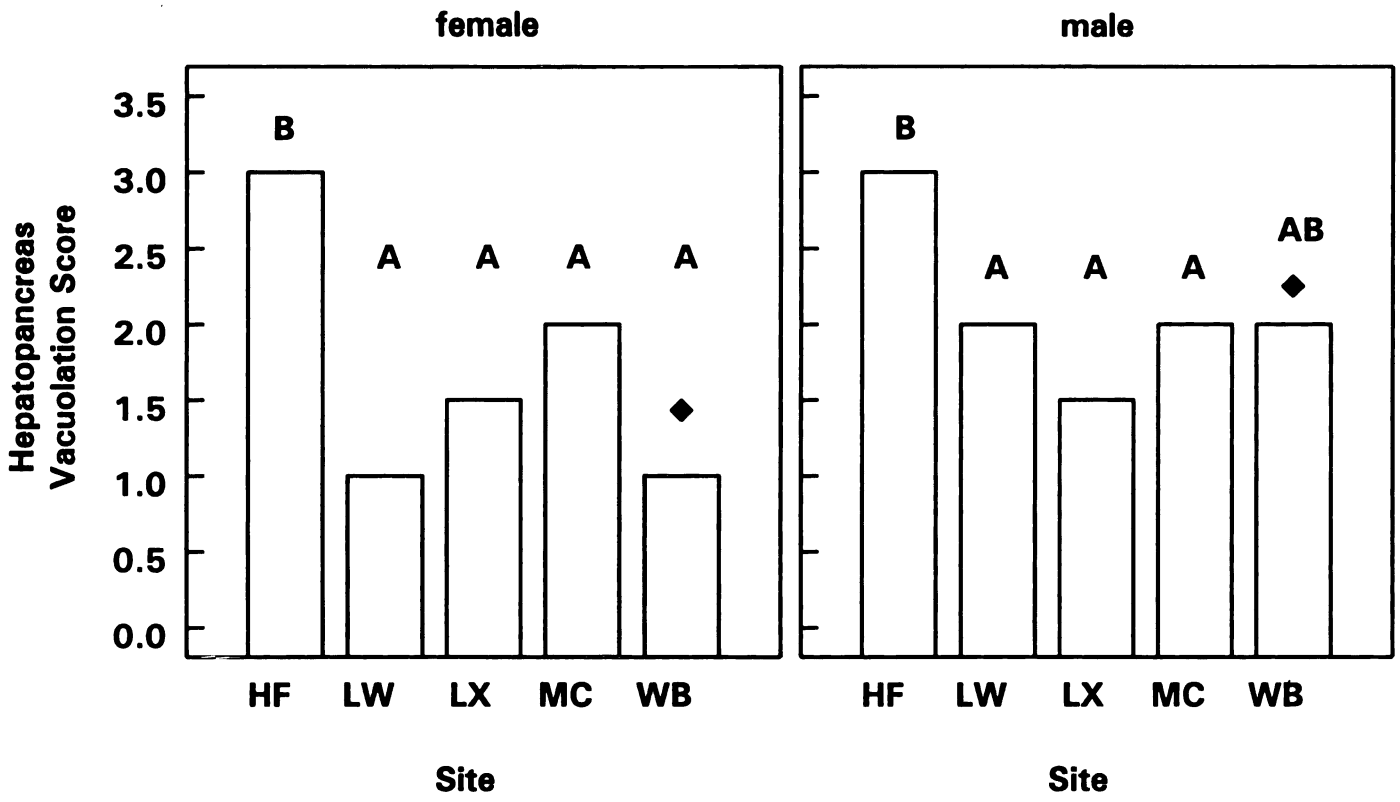

Figure 2-3. Vacuolation of the hepatopancreas in adult female and male carp. Bar height represents median vacuolation score for each site. Letters above the bars represent Tukey groupings within each sex. ♦ = significant difference in median vacuolation score from fish of the opposite sex caged at the same site.

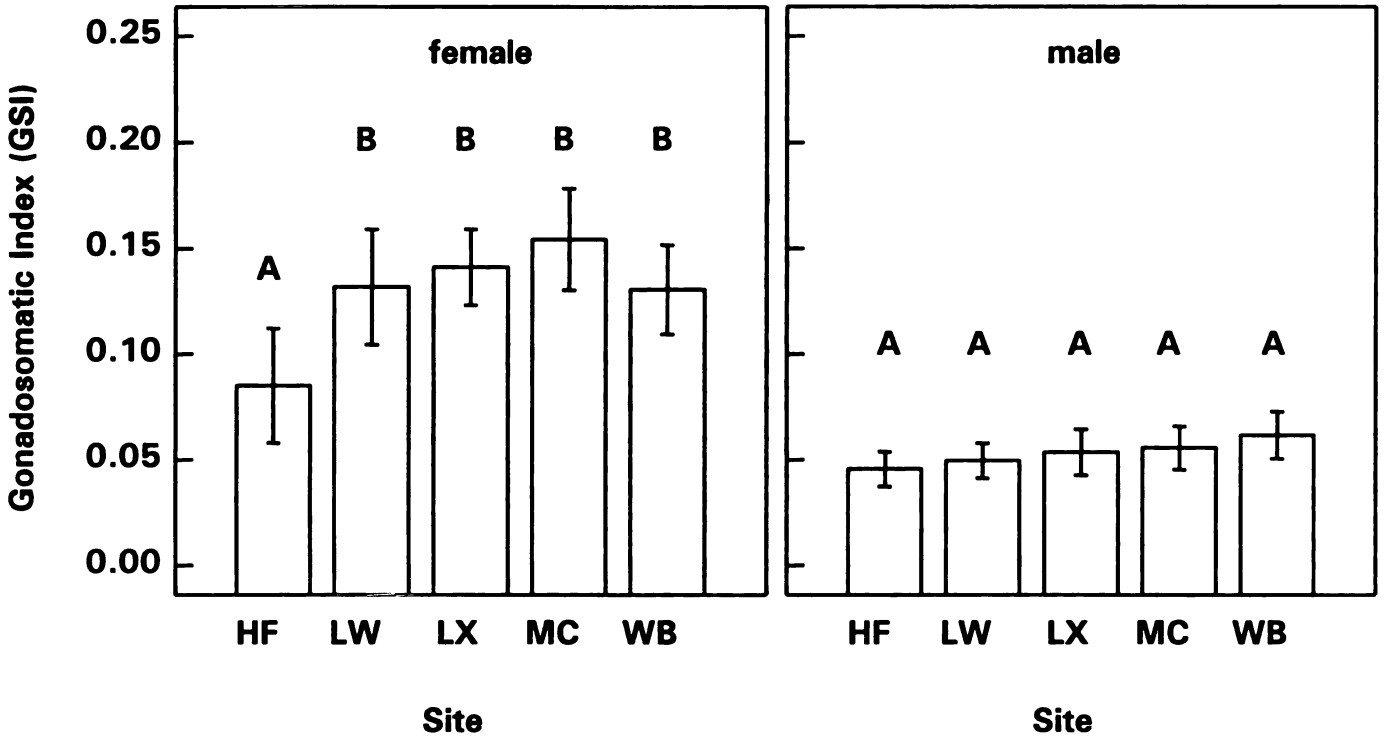

Figure 2-4. Gonadosomatic index (GSI). Bar height represents mean, and error bars represent 2 SEM. Letters above the bars represent Tukey groupings within each sex. There were no significant differences in mean GSI among male carp at different sites.

$$GSI = \frac{\text{gonad weight}}{(\text{body weight - gonad weight})}$$ where all weights are in grams.

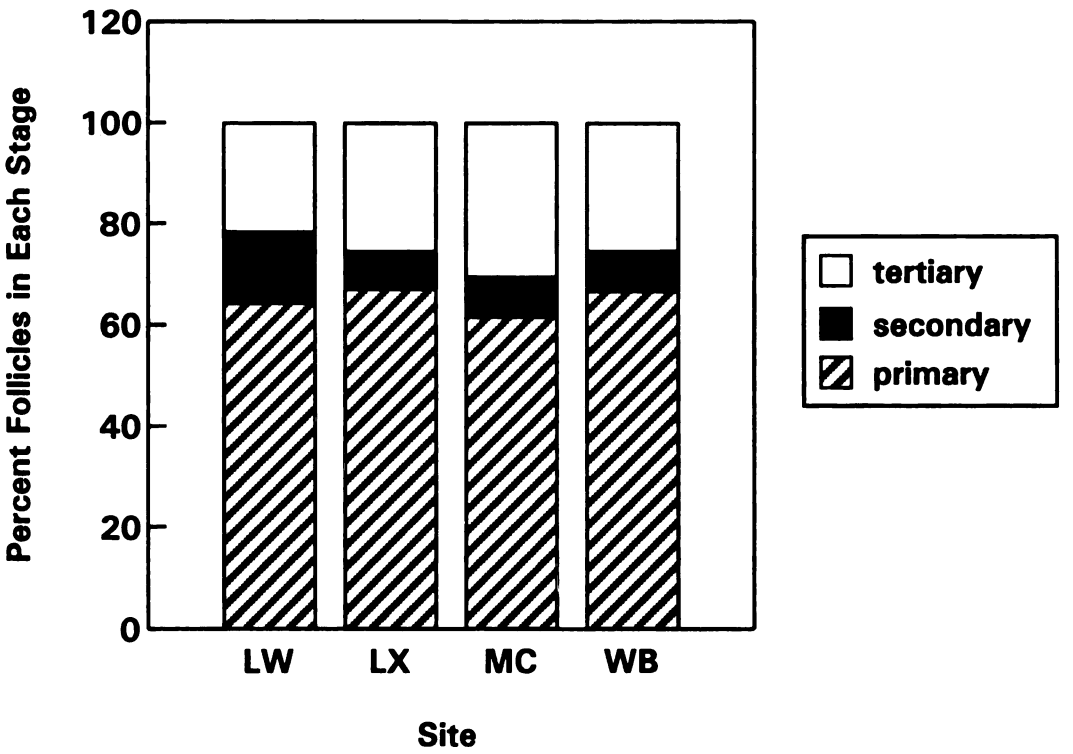

Figure 2-5. Percentages of ovarian follicles in each of three stages: primary, secondary, and tertiary. Bar segments represent the mean percentage of follicles in each stage.

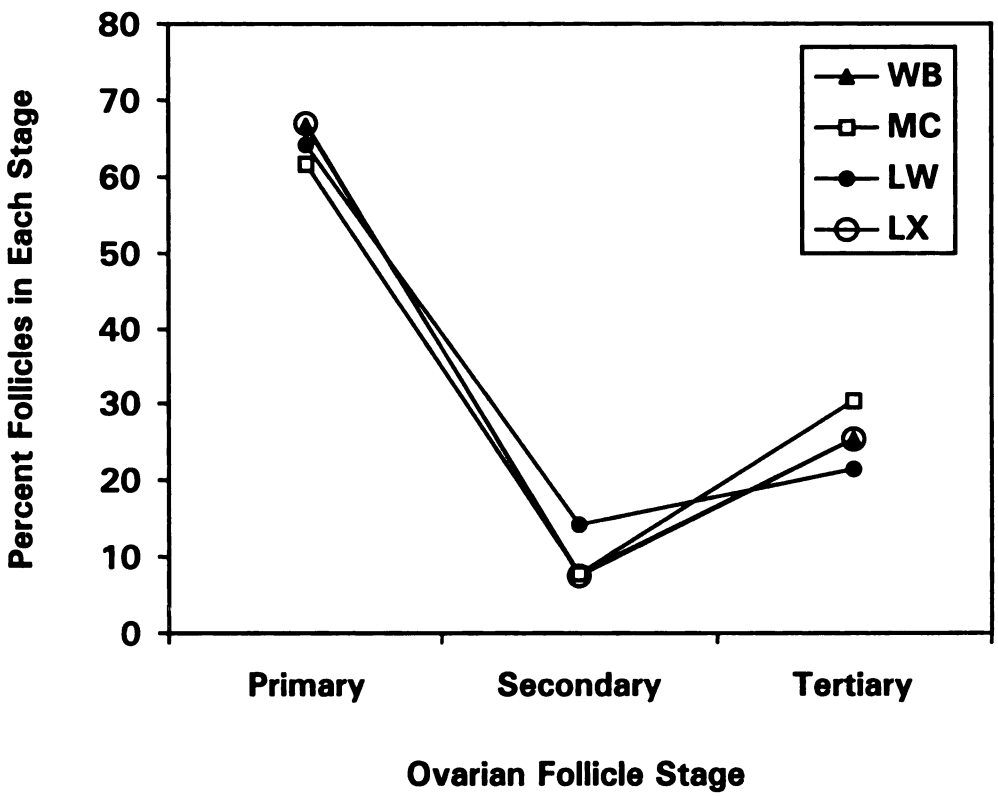

Figure 2-6. Profiles of percentages of ovarian follicles in each of three stages: primary, secondary, and tertiary. Each point represents the mean percentage of follicles in the corresponding stage.

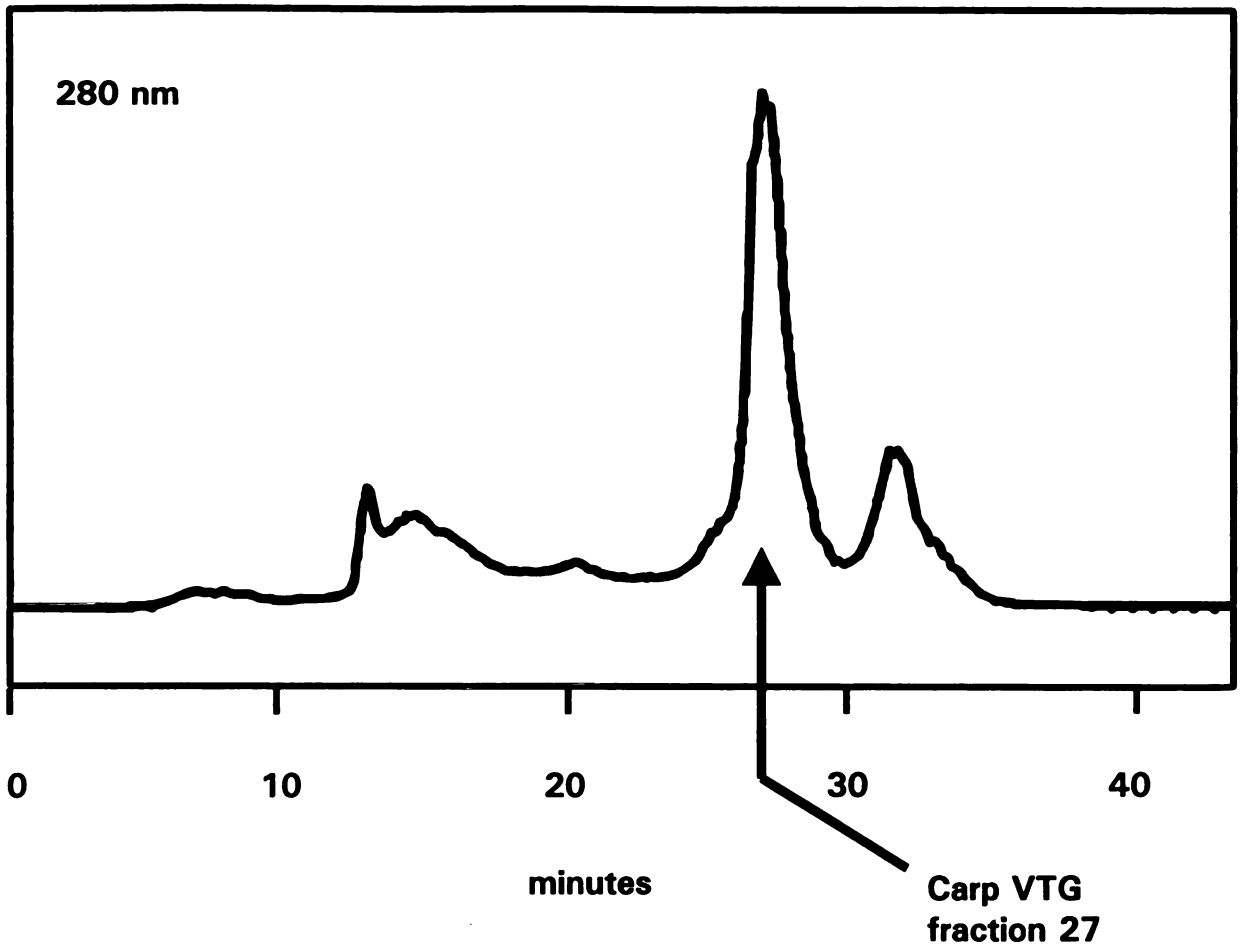

Figure 2-7. HPLC chromatogram for common carp vitellogenin (VTG). Carp VTG was purified on a DEAE anion exchange column. Fraction 27 represents carp VTG.

Figure 2-8. Western blot of purified common carp and goldfish vitellogenin (VTG) standard solutions and male and female carp plasma samples. mw = molecular weight standards, gs = purified goldfish VTG standard, cs = purified carp VTG standard, fc = female carp plasma, mc + = E2-induced male carp plasma, mc- = uninduced male carp plasma, mp = pooled plasma from uninduced male carp. E2 = 17β-estradiol.

mw
gs
cs
fc
mc+
mc-
mp
mw

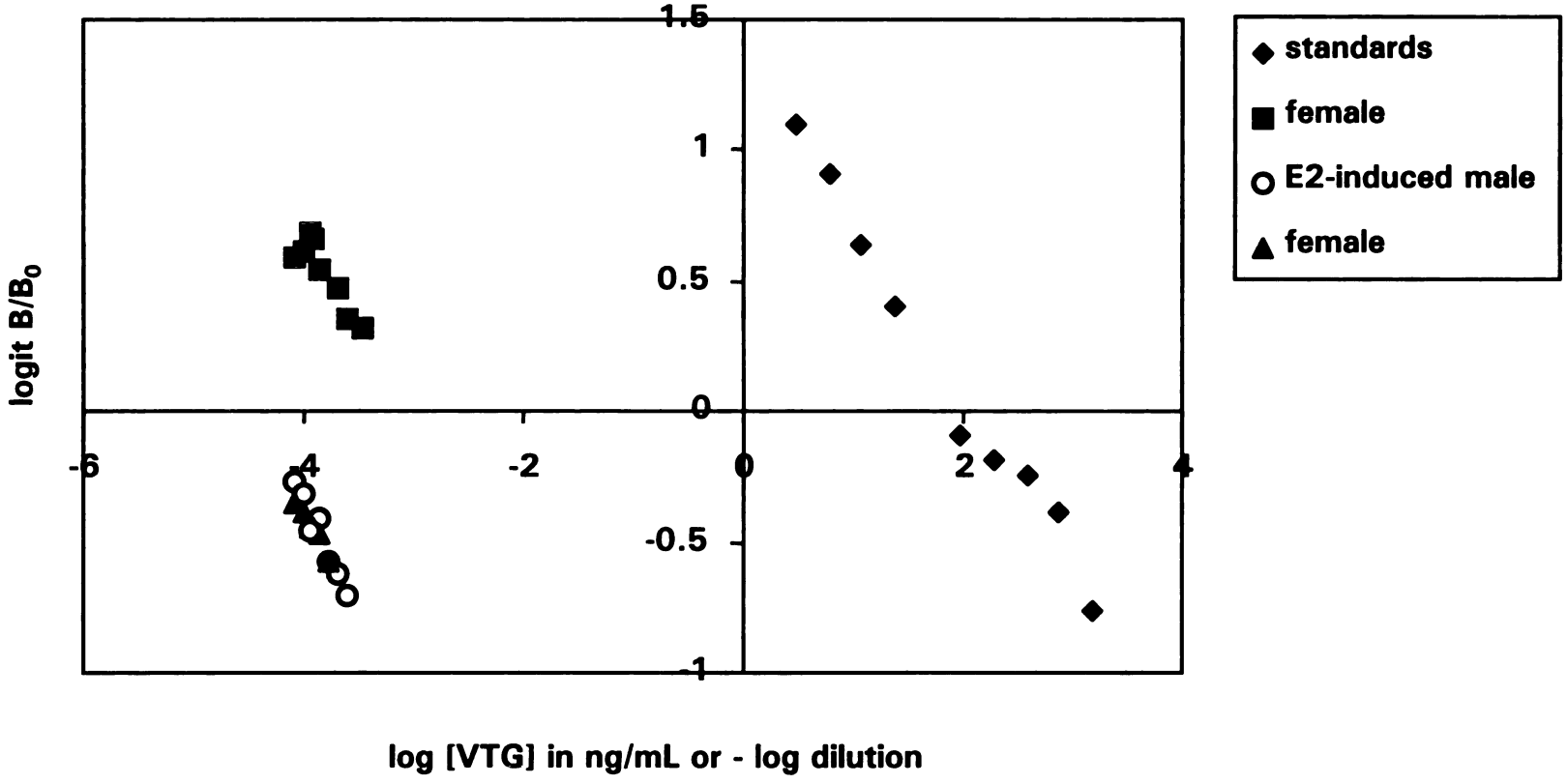

Figure 2-9. Dilution curves for vitellogenin (VTG) in female carp plasma and E2-induced male carp plasma demonstrating parallelism with purified carp vitellogenin (VTG) standard in a VTG enzyme-linked immunosorbent assay (ELISA). E2 = 17β-estradiol, logit B/B_0 = logit-transformed % bound, log [VTG] = log concentration of VTG, - log dilution = - log dilution factor for carp plasma. Parallelism of the dilution curves and standard curve was demonstrated by analysis of covariance.

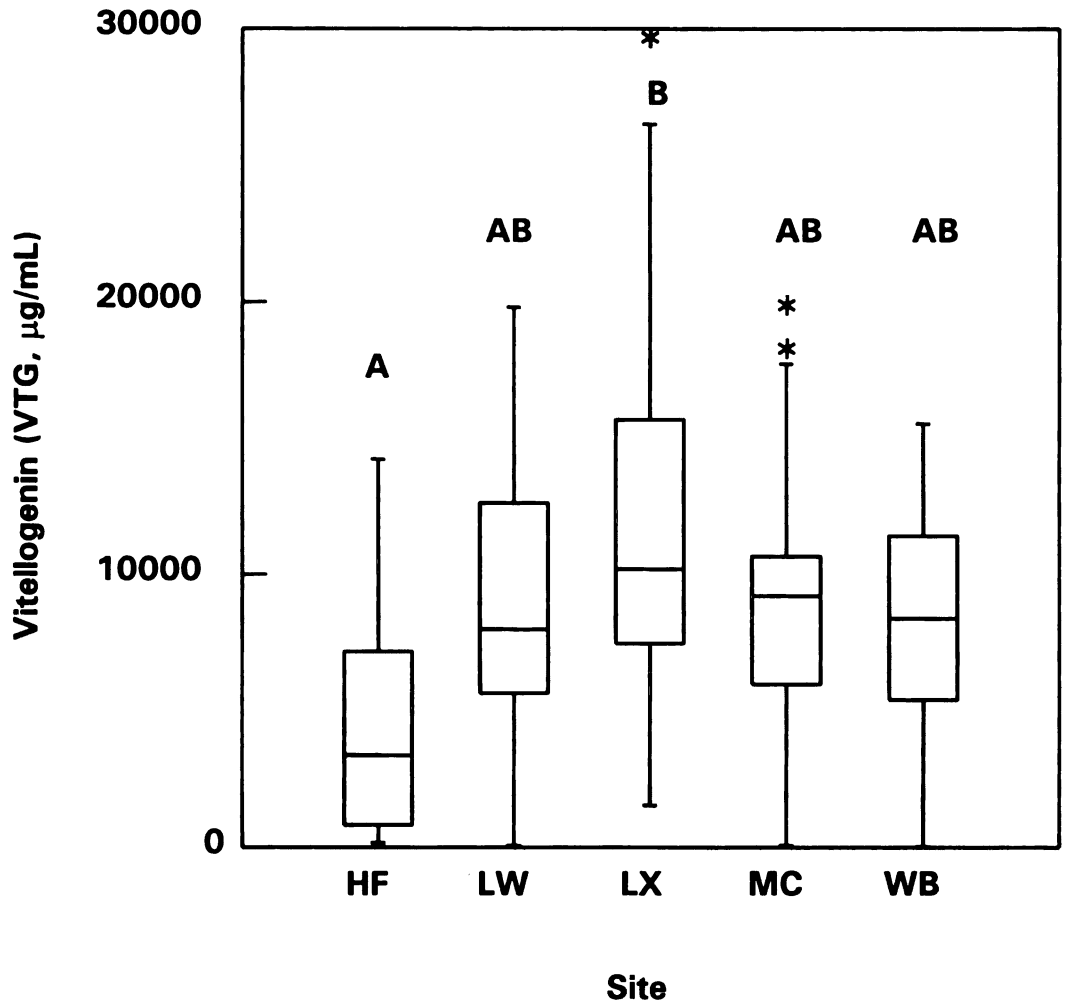

Figure 2-10. Plasma vitellogenin (VTG, µg/mL) concentrations in female carp. Letters above boxes represent Tukey-like groupings. The center horizontal lines mark the medians for each site, and upper and lower edges of the boxes (hinges) represent first and third quartiles. Whiskers show the range of observed values that fall within the inner fences. Asterisks mark "outside values" (those between the inner and outer fences). See text under **"Statistical analyses"** for discussion of terms.

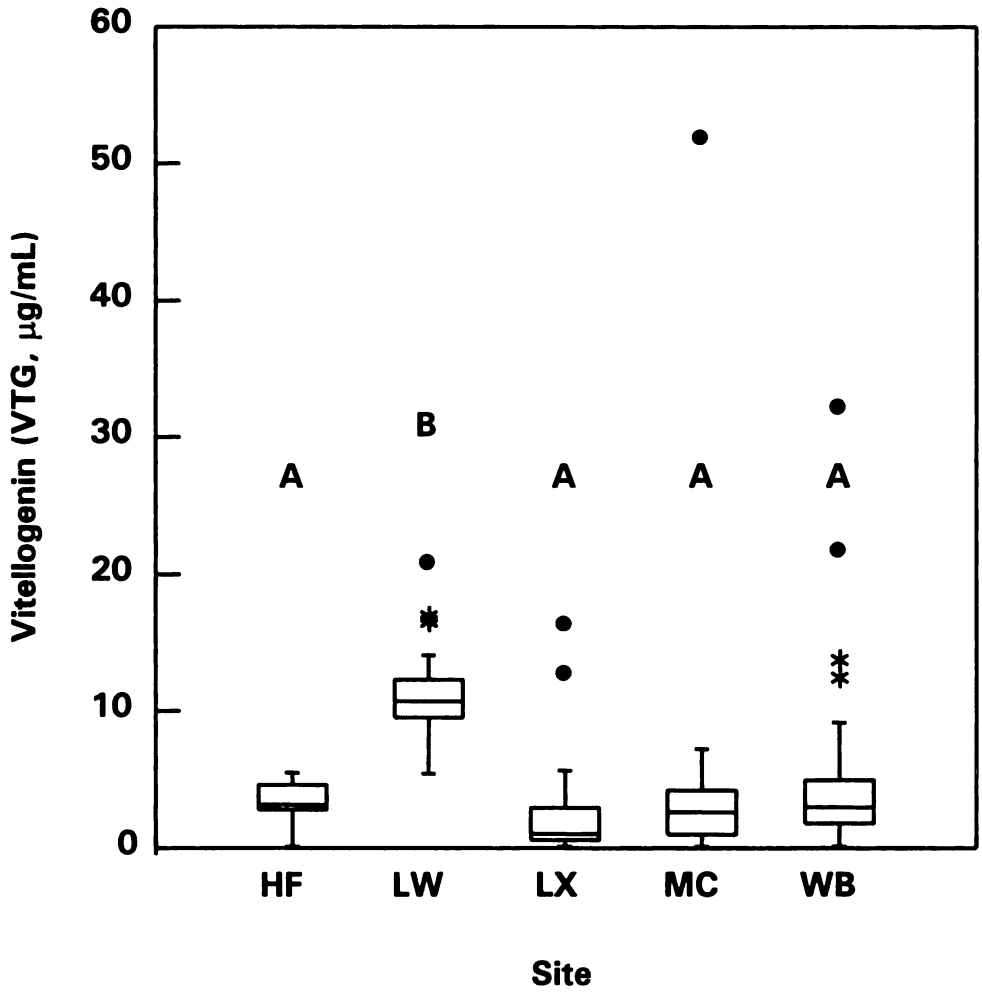

Figure 2-11. Plasma vitellogenin (VTG, µg/mL) concentrations in male carp. Letters above boxes represent Tukey-like groupings. The center horizontal lines mark the medians for each site, and upper and lower edges of the boxes (hinges) represent first and third quartiles. Whiskers show the range of observed values that fall within the inner fences. Asterisks mark "outside values" (those between the inner and outer fences), and circles mark "far outside values" (those beyond the outer fences). See text under "**Statistical analyses**" for discussion of terms. Note the difference in the scale of the y-axis relative to that for females in Figure 10.

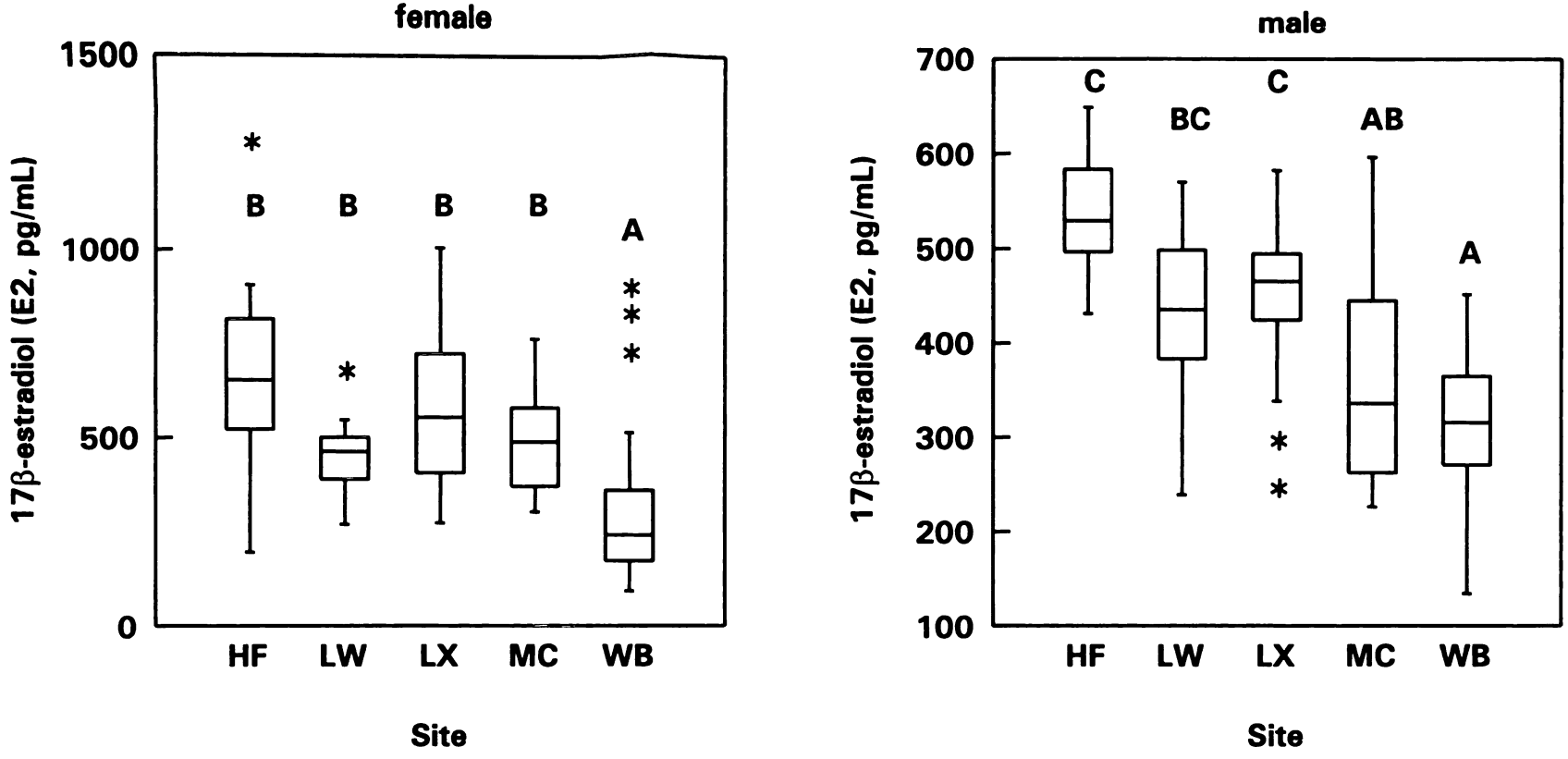

Figure 2-12. Plasma 17β-estradiol (E2, pg/mL) concentrations in adult male and female carp. Letters above boxes represent Tukey-like groupings within each sex. The center horizontal lines mark the medians for each site, and upper and lower edges of the boxes (hinges) represent first and third quartiles. Whiskers show the range of observed values that fall within the inner fences. Asterisks mark "outside values" (those between the inner and outer fences). See text under **"Statistical analyses"** for discussion of terms. The sexes differed significantly in median plasma E2 concentration within a site only at MC.

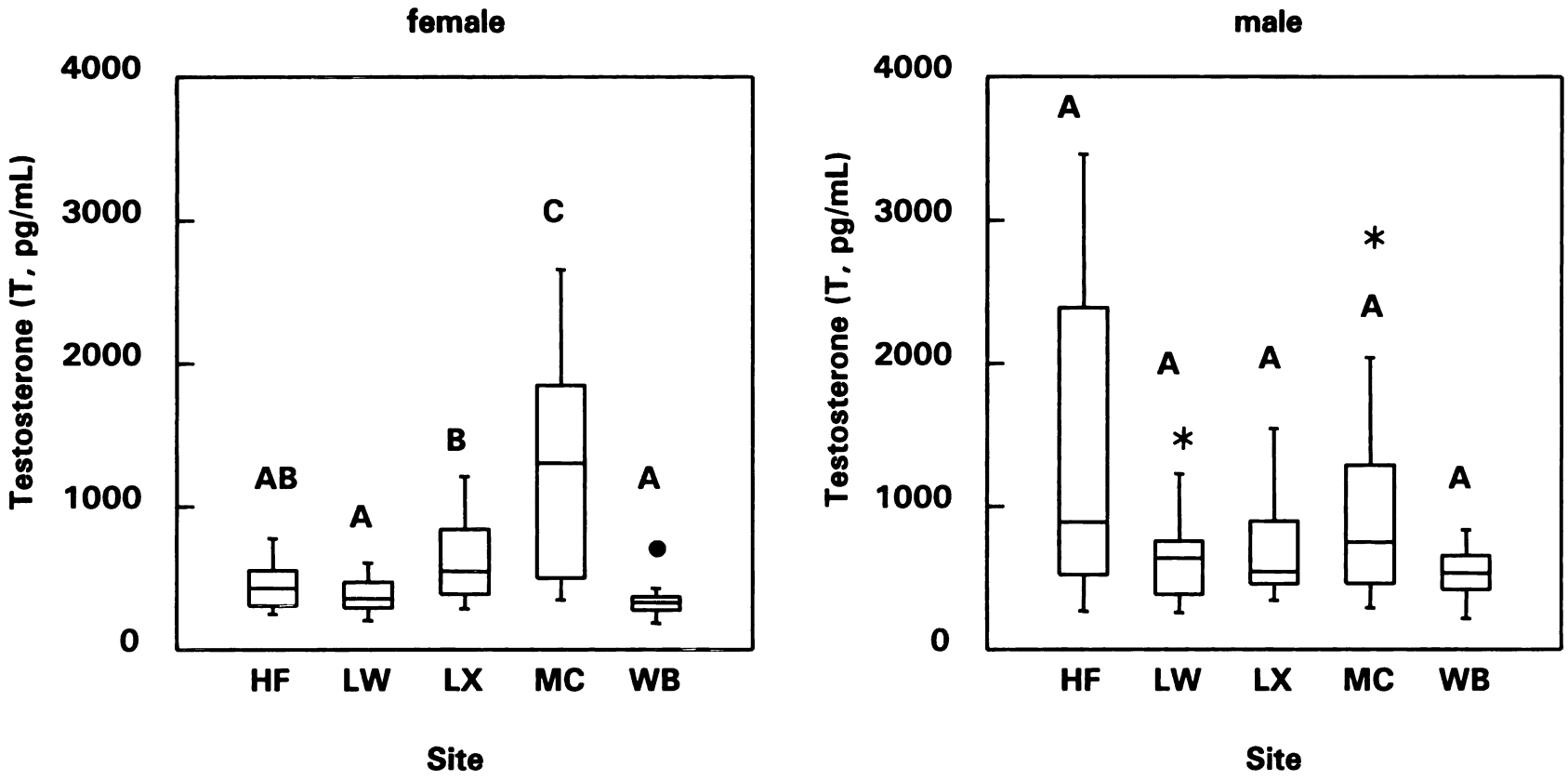

Figure 2-13. Plasma testosterone (T, pg/mL) concentrations in adult female and male carp. Letters above boxes represent Tukey-like groupings within each sex. The center horizontal lines mark the medians for each site, and upper and lower edges of the boxes (hinges) represent first and third quartiles. Whiskers show the range of observed values that fall within the inner fences. Asterisks mark "outside values" (those between the inner and outer fences), and circles mark "far outside values" (those beyond the outer fences). See text under **"Statistical analyses"** for discussion of terms. The sexes differed significantly within sites at HF, LW, and WB.

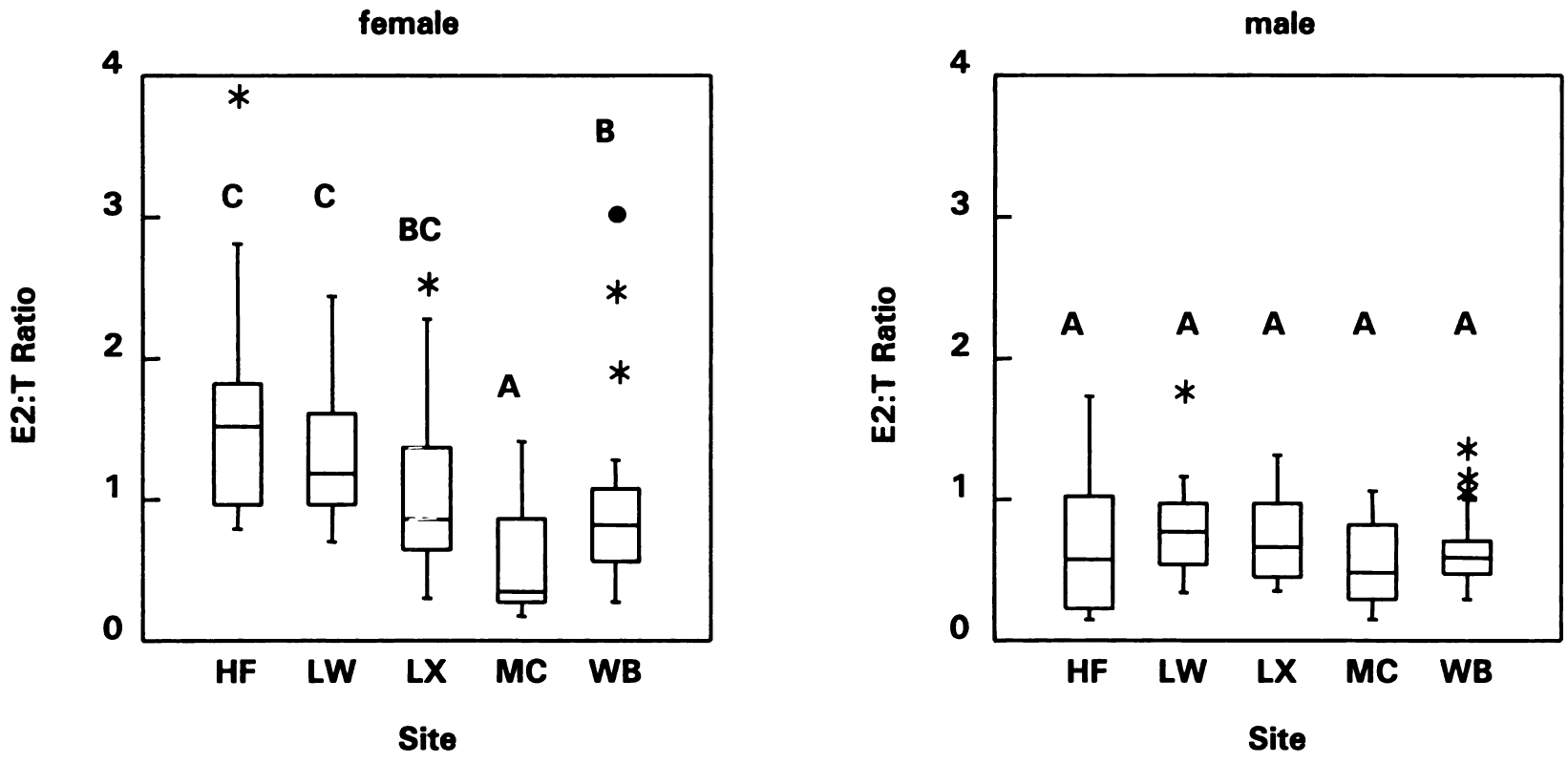

Figure 2-14. Ratio of plasma 17β-estradiol to plasma testosterone (E2:T) in adult female and male carp. Letters above boxes represent Tukey-like groupings within each sex. The center horizontal lines mark the medians for each site, and upper and lower edges of the boxes (hinges) represent first and third quartiles. Whiskers show the range of observed values that fall within the inner fences. Asterisks mark "outside values" (those between the inner and outer fences), and circles mark "far outside values" (those beyond the outer fences). See text under **"Statistical analyses"** for discussion of terms. The sexes differed significantly in site mean or median E2:T among fish caged at the same site only at HF and LW.

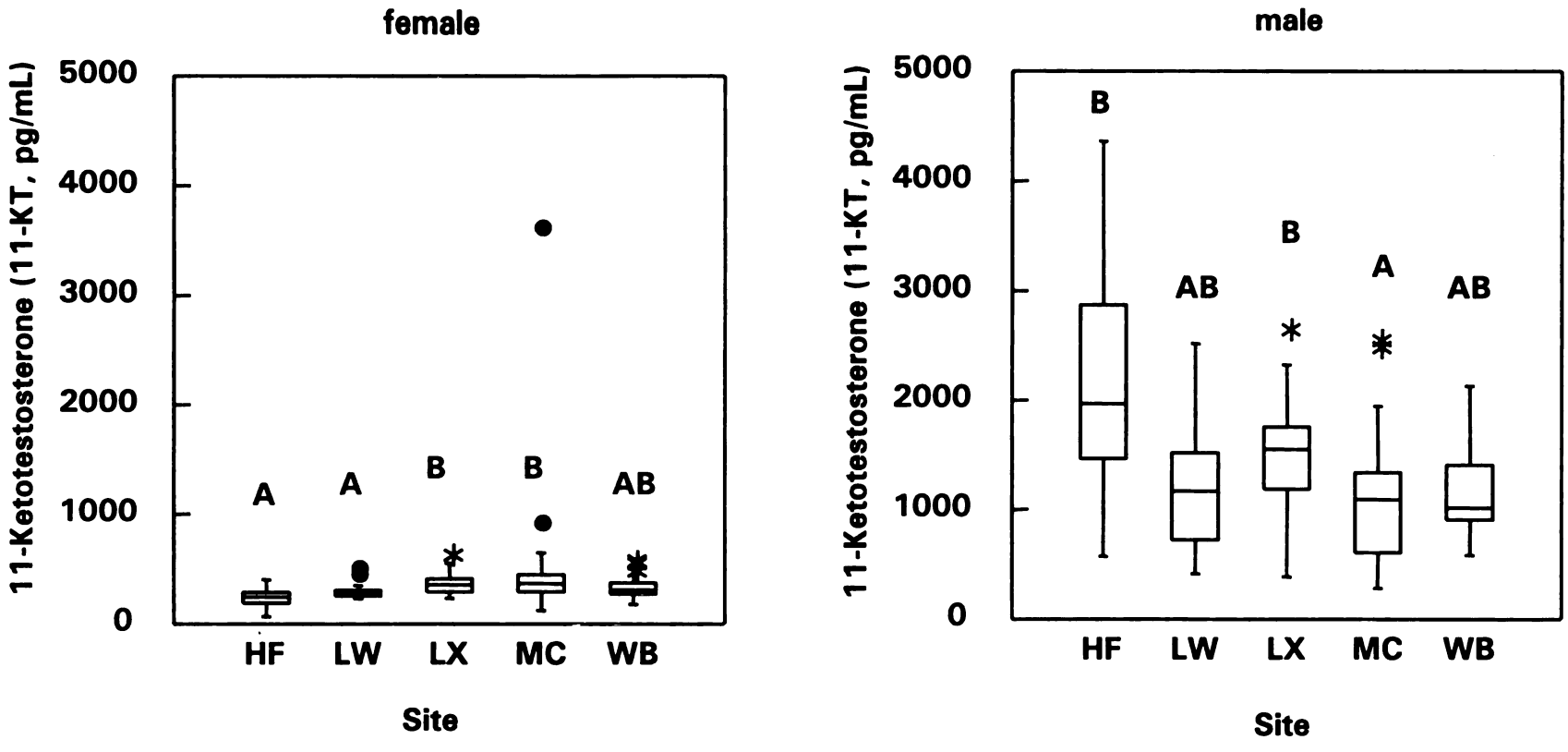

Figure 2-15. Plasma 11-ketotestosterone (11-KT, pg/mL) concentrations in adult female and male carp. Letters above boxes represent Tukey-like groupings within each sex. The center horizontal lines mark the medians for each site, and upper and lower edges of the boxes (hinges) represent first and third quartiles. Whiskers show the range of observed values that fall within the inner fences. Asterisks mark "outside values" (those between the inner and outer fences), and circles mark "far outside values" (those beyond the outer fences). See text under **"Statistical analyses"** for discussion of terms. Males and females differed significantly in median plasma 11-KT concentration within each site.

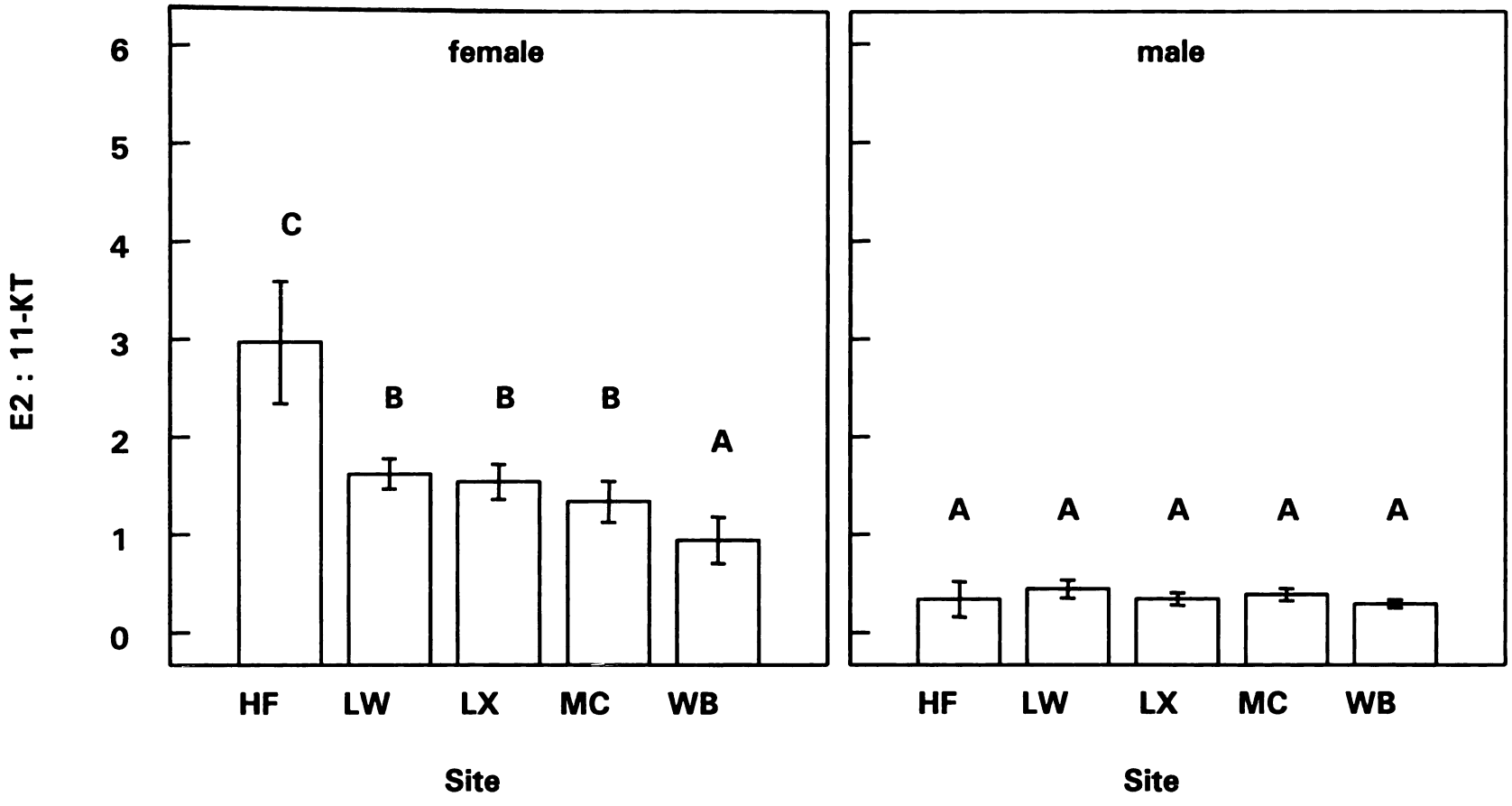

Figure 2-16. Ratio of plasma 17β-estradiol to plasma 11-ketotestosterone (E2:11-KT) in adult female and male carp. Letters above bars represent Tukey groupings within each sex. The heights of the bars represent site means. Error bars are 2 SEM. Males and females caged at the same site differed significantly in plasma E2:11-KT at every site.

Chapter 3

EVALUATION OF REPRODUCTIVE ENDPOINTS IN COMMON CARP (*CYPRINUS CARPIO*) CAGED *IN SITU* AT SITES RECEIVING WASTEWATER FLOW IN LAKE MEAD, NEVADA, (APRIL TO MAY)

(Intended for submission to *Environmental Toxicology and Chemistry*)

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ABSTRACT

Recent reports have indicated that chemical contaminants entering Lake Mead, Nevada, via the Las Vegas Wash are capable of causing estrogenic effects in wild common carp and in an estrogen-responsive MCF7-luciferase cell bioassay. Adult male and female common carp (*Cyprinus carpio*) were exposed separately in cages (30 fish per cage) for 38 to 49 d at four sites in Lake Mead: one site directly in the influent of the Las Vegas Wash as it enters Lake Mead, one site in Las Vegas Bay where the influent of the Wash is more dilute, and two reference sites. Exposures took place from April to May. Endpoints examined included condition factor (K), gonadosomatic index (GSI), plasma concentrations of sex steroids and vitellogenin (VTG), and histology of the hepatopancreas, ovary, and testis. Plasma sex steroids measured were 17β-estradiol (E2), testosterone (T), and 11-ketotestosterone (11-KT). A study nearly identical to the one reported here was conducted earlier in the year (March to April). In that previous study, the median plasma VTG concentration in male carp caged in the Las Vegas Wash was elevated 3- to 10-fold above the concentrations measured in male carp at all other sites, possibly due to exposure to an exogenous estrogenic substance. The results of the current study are somewhat different from the results of our previous study. No significant VTG induction was observed in male carp. However, the hypothesis that the mild (statistically non-significant) Sertoli cell proliferation observed in male carp exposed to Las Vegas Wash

influent in the earlier study was related to site was supported by the finding of increased Sertoli cell proliferation in the testes of male carp caged in Las Vegas Bay in the current study. It cannot be concluded at this time that Sertoli cell proliferation is caused exclusively by exposure to estrogenic substances, though, and the lesions observed were mild. Similar to the results of the initial study, there are no effects on plasma steroid hormone profiles related to the Las Vegas Wash influent that do not have potential explanations rooted in site physical characteristics or differences in reproductive timing. Most importantly, it appears that the Las Vegas Wash influent might have inhibited testicular growth in male carp exposed to the influent in the Las Vegas Wash and in Las Vegas Bay and decreased plasma 11-KT concentrations in male carp caged at LW. The finding of decreased plasma 11-KT concentration in male carp exposed to Las Vegas Wash influent is consistent with effects reported in wild male carp caught in Las Vegas Bay by USGS researchers. However, because water temperatures at the two sites potentially were great enough to induce spawning in carp in the current study, the findings of reduced GSI and plasma 11-KT concentrations in the current study are tentative.

INTRODUCTION

Lake Mead is a large reservoir formed by impoundment of the Colorado River behind the Hoover Dam. The reservoir serves as a source of domestic and agricultural water for more than 22 million users (LaBounty et al., 1997) and, as part of the Lake Mead National Recreational Area, is used for activities such as boating, swimming, and fishing. The Las Vegas Wash enters the Boulder Basin of Lake Mead via the Las Vegas Bay (Figure 3-1) and provides one the greatest inflows of water into Lake Mead, second only to the Colorado River (LaBounty et al., 1997). The flow of the Las Vegas Wash consists entirely of all tertiary treated municipal sewage effluent, urban storm water flow, and groundwater seepage from the urbanized Las Vegas Valley (LaBounty et al., 1997). The water entering Lake Mead via the Las Vegas Wash is considerably more dense and exhibits greater conductivity and turbidity than main body Lake Mead water (LaBounty et al., 1997). The difference in density causes the intrusion of the Las Vegas Wash to form an interflow that extends from Las Vegas Bay into the Boulder Basin. At times, the influence of this interflow has been detected on the basis of its conductivity at the Hoover Dam, 16 km away from the confluence of the Las Vegas Wash with Lake Mead.

In 1996, the United States Geological Survey (USGS) reported evidence that suggested endocrine disruption in wild adult male and female common carp

(*Cyprinus carpio*) associated with the Las Vegas Wash and Bay (Bevans et al., 1996). This evidence included alterations in plasma sex steroid concentrations in male and female fish relative to those observed in fish at a reference site and induction of a female-specific protein, vitellogenin (VTG), in the blood of male carp. These findings prompted concern for the razorback sucker (*Xyrauchen texanus*), an endangered species of fish that spawns in the Las Vegas Bay (Holden et al., 1999).

The potential causes of the effects observed in the Lake Mead carp are many, but the induction of VTG in male fish without a concomitant increase in plasma E2 indicated that the fish had been exposed to an estrogen-like (estrogenic) chemical in the environment. VTG is an egg yolk precursor synthesized in the liver of fish in response to the estrogenic sex steroid 17β-estradiol (E2). VTG is transported in the blood to the ovary, where it is sequestered into developing oocytes in preparation for spawning (Mommsen et al., 1988). VTG serves no known function in male fish and normally is not detected (or detected only in very low concentrations) in their blood plasma, presumably because E2 levels are not great enough in male fish to induce VTG synthesis (Mommsen et al., 1988; Specker et al., 1994). However, male fish possess the VTG gene and are capable of producing VTG when exposed to exogenous estrogenic chemicals (Mommsen et al., 1988; Specker et al., 1994). VTG is a useful biomarker of exposure to estrogenic

chemicals in male fish for several reasons. VTG induction is a specific response to estrogenic chemicals, and, in male fish, is attributable to exposure to an estrogenic substance (Mommsen et al., 1988). Because male fish do not possess a normal clearance mechanism for VTG (i.e., deposition into oocytes), it can build to considerable and measurable concentrations in their blood plasma (Mommsen et al., 1988). Also, only a small volume of plasma is needed for measurement of VTG, leaving the remainder for measurement of plasma sex steroids or other hormones that might be important in evaluating potential reproductive dysfunction.

Exposure to an estrogenic chemical also might cause alterations in plasma sex steroid levels in fish, although not necessarily through an estrogen receptor-mediated mechanism (MacLatchy et al., 1997; Tremblay et al., 1998). Given that the Las Vegas Wash contains wastewater, which can be expected to contain a complex mixture of chemical contaminants, the effects on sex steroids and on plasma VTG in Lake Mead carp might have occurred through entirely different mechanisms and/or by exposure to different chemicals. Studies in the United Kingdom (UK) (Harries et al., 1996; Harries et al., 1997; Harries et al., 1999) and in the United States (US) (Folmar et al., 1996) on fish in municipal sewage effluent or in rivers receiving it have suggested that contaminants in sewage effluent can cause VTG induction and other reproductive effects in fish. Results of the work conducted in the

UK indicated that VTG induction observed in fish in sewage effluents and in some rivers might be caused by water soluble contaminants such as the animal steroids E2 and estrone (E1) and/or an oral birth control medication component, ethinylestradiol (EE2) (Routledge et al., 1998).

To investigate the possibility that water soluble contaminants in the Las Vegas Wash might be causing estrogenic effects in Lake Mead fish, researchers from Michigan State University (MSU) adopted a toxicity identification and evaluation (TIE) scheme. In 1997, water samples were collected from LW, LX, and two reference sites in Lake Mead (Saddle Island and Callville Bay) and subjected to screening for estrogenic substances by analytical chemistry and *in vitro* bioassay techniques (Snyder et al., 2000b). Significant estrogenic responses were induced in the bioassay by extracts from LW and LX water samples and not by extracts from the reference sites. The results of the screening indicated that steroidal estrogens such as E2 and EE2 were likely causes for the observed estrogenic bioactivity of the LW and LV extracts *in vitro* (Snyder et al., 2000b).

Although steroidal estrogens appeared to be the most potent estrogenic chemicals in extracts from LW and LX when tested *in vitro*, it cannot be assumed that this would hold true *in vivo* as well. Estrogens produce a variety of effects at multiple targets, some of which involve interaction of

different tissues; these types of effects cannot be predicted with a single *in vitro* bioassay (Zacharewski, 1997). *In vitro* bioassays do not account for pharmacokinetics, bioaccumulation, or metabolism (Zacharewski, 1997), all of which can cause substantial differences in the responses of whole animals versus cells in culture. Therefore, the study reported here was intended to address the following questions: (1) Can effects observed in wild carp by researchers at USGS be reproduced in caged fish? (2) Are steroidal estrogens and other relatively polar contaminants present in the Las Vegas Wash and Las Vegas Bay at concentrations likely to cause reproductive effects observed in fish? For several reasons, this study complements the ongoing USGS work with wild carp. The wild carp represent fish receiving natural exposure. Wild carp will feed on detritus in the lake sediment and might be exposed to more hydrophobic chemicals present in food or sediment by this route. They also might receive longer-term exposure or exposure during critical periods of development that might result in effects that would not be detected with a short-term exposure conducted with adult fish. However, the caged fish receive a more controlled exposure with a specified duration and under monitored conditions. Wild fish are free to move in and out of the influence of the Las Vegas Wash, whereas caged fish receive a known level of exposure.

METHODS AND MATERIALS

Fish

Sexually mature, adult male and female common carp (2 to 3 yr of age) were purchased from J&J Aquafarms (Sanger, California). Male and female fish were held in separate cement holding ponds at the Lake Mead Fish Hatchery, Boulder City, Nevada, for the shortest length of time possible (2 to 11 d) prior to placing them into cages in the field. Although a longer acclimation period is preferable, outbreaks of *Ichthyophthirius multifilis* ("Ich") in the hatchery fish are common even when fish are stocked under optimal conditions, and a longer acclimation period might have required prophylactic treatment with chemicals that could affect the endpoints of interest. While fish were held at the hatchery, they were fed daily to satiation with a floating dense culture pellet food for pond fish (Aquatic Ecosystems, Apopka, Florida; F2G). A floating pellet formulation was used so that fish could be observed at the surface for normal feeding behavior and health. Fish were not fed the day prior to placing them in cages.

Cage deployment

Fish cage kits were purchased from Aquatic Ecosystems (Apopka, Florida; catalog number C3). Cages were constructed of a polyvinyl chloride (PVC) pipe frame and polypropylene knotless mesh in order to prevent injury to the fish. Cage dimensions were 2.1 m X 2.1 m X 1.4 m deep. Cages were

suspended approximately 1.4 to 1.7 m below the surface of the water as described previously (Snyder et al., 2000a).

Two cages, one containing 30 males and one containing 30 females, were deployed at each of four sites in Lake Mead (Figure 3-1). Two sites, Water Barge Cove (WB) and Moon Cove (MC), are not influenced significantly by the flow of the Las Vegas Wash and could be considered reference sites. Two cages were placed directly at the point where the Las Vegas Wash enters the Las Vegas Bay such that the fish at this site received maximal exposure to the influent. This site was named Las Vegas Wash (LW). The remaining two cages were placed further out in Las Vegas Bay at a point where the flow entering from the Wash was more dilute but still readily detected by greater conductivity and turbidity than was observed in main body lake water. All sites were monitored for conductivity and other water quality parameters (see below), in part to ensure that the fish were receiving the expected exposure to the flow of the wash.

Initial total weight of carp placed in each cage was determined by weighing a bucket of water on a scale, then adding the fish and re-weighing. The initial mean weight was calculated by dividing the initial total weight by the number of fish weighed and converting to mean weight in grams per fish. Fish were transported by boat in aerated livewells to the exposure sites.

Exposure site monitoring

Monitoring was conducted as described previously (Snyder et al., 2000a). Fish were fed approximately twice weekly or as weather permitted by dropping a sinking pellet food (Silver Cup trout pellets; Nelson & Sons, Inc.; Murray, Utah) through the top of each cage. Fish were observed to be feeding at the reference sites but could not be seen at sites LX and LW due to water turbidity. Water quality at all sites was monitored as described previously (Snyder et al., 2000a).

Cage retrieval and sample collection

Cages were retrieved and samples collected as described previously (Snyder et al., 2000a). Briefly, two fish cages (one for each sex) were retrieved from one site per sampling day. Cage retrievals at the different sites were conducted over the shortest period of time possible to minimize variation in the endpoints of interest. Fish were collected from the cages and placed into aerated livewells for transport a short distance back to a dock for sampling. At the dock, fish were killed by overdose of approximately 200 mg/L tricaine methanesulfonate (Tricaine-S or MS-222; Aquatic Ecosystems, Apopka, Florida; TRS1). Pressure was applied to the abdomen of male fish to determine whether milt was present. Blood was collected immediately and quickly from the caudal vasculature of each fish using a 3 mL heparinized syringe and 20- or 22-gauge needle. Up to 3 mL blood was

placed into centrifuge tubes pretreated with the protease inhibitor aprotinin (Sigma, St. Louis, Missouri; A-1153), then placed on ice for transport back to the laboratory. Each carcass was placed into an individual labeled bag and transported on ice back to the laboratory for dissection. In order to minimize variation in plasma hormone levels caused by diel cycling, fish of the same sex at different sites were consistently sampled for blood within the same 2- to 3-hr time period (McMaster et al., 1992).

In the laboratory, the abdominal cavity of each fish was opened to confirm the sex by observation of the gonads. In the interests of time and obtaining fresh samples, a random subsample of 16 fish from each cage per site (16 fish of each sex) was selected for collection of gonad weights and histology samples. Gonads were removed and weighed for calculation of gonadosomatic index (GSI), where GSI = [(gonad weight)/ (total body weight - gonad weight)] and all weights are in g. Gonad and hepatopancreas samples were collected and preserved in neutral-buffered formalin for later histological examination. At the laboratory, blood samples were centrifuged at 3000 × g for 10 min at 4 °C. Plasma was collected, divided into several aliquots per sample, and frozen at -80 °C until analysis for VTG and sex steroids.

Final total weight was calculated by summing the individual weights of fish recorded when the fish were retrieved and dividing by the number of fish retrieved. Because the method of measurement of initial weight was crude and the measurements of initial and final weights were made in different fashions, comparisons between initial and final weights should be considered as rough estimates. These measurements were made only to determine whether weight, and thus presumably condition, was reduced by stress related to caging. Individual measurements of weight and length were not made on fish prior to caging and because it was necessary to reduce handling stress to the greatest extent feasible. Likewise, weighing the fish as a group at the end of the exposure to obtain a measure analagous to the initial weight determination would have increased handling stress and slowed sample collection during a critical time period. Fulton-type condition factors (K) were calculated from length and weight measurements as $K = (W/L^3) \times 10{,}000$ where W = weight in g and L = length in mm (Anderson et al., 1996).

Histology

Gonad and hepatopancreas samples taken for histological examination were fixed in neutral-buffered formalin and processed as described previously (Snyder et al., 2000a). Sections from the centermost portion of each gonad were trimmed and placed into tissue cassettes. Ovaries were sectioned

transversely and testes were sectioned longitudinally. Tissues were embedded in paraffin, sectioned at 5 μm, and stained with haematoxylin and eosin. All slides were examined by a Board-Certified veterinary pathologist.

Hepatopancreas histologic criteria

Slides of hepatopancreas tissue were examined for signs of necrosis, inflammation, neoplasia, foci of atypia, biliary stasis, and cellular degeneration. Vacuolar degeneration of hepatocellular cytoplasm (hepatocellular vacuolation) is a nonspecific lesion and indicates accumulation of glycogen and/or fat due to overfeeding, emaciation (use of fat stores), toxification due to various substances, and other causes. Hepatocellular vacuolation was graded on a scale as follows: 0 = no vacuolation, 1 = mild vacuolation with small vacuoles spread throughout the cytoplasm, 2 = moderate vacuolation with larger coalescing vacuoles appearing as large clear zones in many hepatocytes, 3 = severe vacuolation where all or most of the cytoplasm has lost its normal pink coloration due to confluent, large, clear vacuoles.

Ovary histologic criteria

Stages of ovarian follicle development were assessed as described previously (Table 3-1) (Miles-Richardson et al., 1999b). A typical area of the ovary was selected, and 50 follicles were counted within that area. Proportions of

primary, secondary, tertiary, and atretic follicles per 50 follicles are recorded. Atretic follicles are unovulated follicles undergoing atresia, a process of degeneration.

Testis histologic criteria

Sections of testis were scanned at 50X and 100X magnifications, and the relative number and prominence of Sertoli cells in comparison to control fish were estimated using the following scale: 0 = no proliferation, 1 = mild proliferation (<one third), 2 = moderate proliferation (one third to two thirds), 3 = severe proliferation (> two thirds). Sertoli cell proliferation previously was observed in male fathead minnows exposed to the natural estrogen 17β-estradiol (E2) or to the estrogenic chemical 4-nonylphenol (Miles-Richardson et al., 1999a; Miles-Richardson et al., 1999b). Sections of testis were examined for the presence of degenerative changes such as germ cell syncytia, mineralization of spermatozoa, and variably sized or necrotic spermatozoa (Miles-Richardson et al., 1999a; Miles-Richardson et al., 1999b). Testes also were designated as spermatogenically active or inactive.

Plasma vitellogenin (VTG)

Plasma samples were analyzed for VTG with a competitive enzyme-linked immunosorbent assay (ELISA) technique previously developed at Michigan

State University (Nichols, 1997) for use with goldfish and fathead minnow plasma samples and later modified for measurement of plasma VTG in carp (Snyder et al., 2000a) by using purified carp VTG as a standard rather than purified goldfish VTG. Other modifications to the original protocol include longer incubation times at lesser temperatures for some steps.

Standards and samples were assayed in duplicate. A percent coefficient of variation was calculated on duplicate OD measurements for each sample, and those that exceeded 10% were re-assayed. Because the log-logit transformation does not function well at the extreme ends of the standard curve, samples that produced less than 15% maximum binding were re-assayed at a greater dilution. A single plasma sample was collected and stored in aliquots in the same fashion as the samples and analyzed twice (two duplicate measurements) on each plate. The average of the final VTG concentrations for the two duplicate analyses was calculated for each plate and used to calculate the inter-assay (inter-plate) percent coefficient of variation (inter-assay %CV) (Grotjan et al., 1996). If r^2 for the standard curve regression line was less than 0.95, the plate was re-run.

Radioimmunoassay of plasma sex steroids

Subsamples of frozen carp blood plasma were shipped on dry ice to the Biotechnology for Evolutionary, Ecological, and Conservation Sciences

Program Laboratory at the University of Florida, Gainesville, for analyses of the plasma sex steroids 17β-estradiol (E2), testosterone (T), and 11-ketotestosterone (11-KT) by competitive radioimmunoassay (RIA) (Goodbred et al., 1997). Prior to RIA analysis, duplicate samples were extracted twice with diethyl ether, evaporated to dryness under nitrogen, and reconstituted in RIA buffer (25 mM sodium phosphate monobasic, 0.05 M sodium phosphate dibasic, 0.15 M sodium chloride, 0.25 g/L sodium azide, 1 g/L gelatin, pH 7.5). Standard curves were prepared by analyzing known concentrations of radioinert steroid diluted in RIA buffer. Cross-reactivities of the E2 antiserum with other female sex steroids were 11.2% for estrone; 1.7% for estriol; < 1.0% for 17α-estradiol and androstenedione; and < 0.1% for ethinylestradiol, diethylstilbestrol, and all other steroids examined. Cross-reactivities of the 11-KT antiserum with other male sex steroids were 9.65% for testosterone, 3.7% for α-dihydrotestosterone, < 1.0 percent for androstenedione, and < 0.1% for all other steroids examined.

Statistical analyses

Data were analyzed with the aid of SYSTAT© Version 9 for Windows (SPSS Science, Chicago, 1998). Where data demonstrated normal distributions and homogeneous variance (homoscedasticity), differences among sites within each sex were examined by one-way analysis of variance (ANOVA) followed by Tukey's HSD post-hoc comparisons of means. When data failed to meet

the assumptions of the parametric statistical methods, a Kruskal-Wallis (nonparametric) test was approximated by examining the ranks of the data by one-way ANOVA followed by a Tukey-like post-hoc comparison of means (Tukey HSD conducted on the ranked data). Normality was assessed by examining probability plots of the normal distributions and by assessing the results of the SYSTAT© Kolmogorov-Smirnov one-sample test with the Lilliefors option (comparison to the standard normal distribution). Because ANOVA testing is robust with respect to departures from the assumptions of homogeneous variance and normal distribution and because nonparametric testing methods have less power to detect differences among groups (Zar, 1984), only severe departures from normality ($p<0.01$) and homoscedasticity were considered sufficient to necessitate the use of nonparametric testing. In most cases, the data were subjected to parametric and nonparametric analyses, and an attempt was made to resolve any differences in the resulting final interpretation. In some cases, $\log_{10}$-transformation normalized the data and decreased the variances; this transformation was used where appropriate. The transformed data were re-examined to determine whether the transformation caused the data to meet the assumptions of parametric statistical methods. If the transformed data met the assumptions, they were subjected to the parametric analyses. In general, outliers were not excluded from analyses, particularly analyses of small data sets ($n<20$), unless this action was justified through best

professional judgement. Outliers were not excluded from charts or graphs with one exception, which is noted in the caption. Exclusion of outliers from statistical analyses is noted in the discussion of the results. Unless otherwise stated, effects were considered to be statistically significant at the 0.05 level of type I error (α) for all analyses, with the exception of tests used to assess normality of distributions (noted previously).

Multivariate profile analysis (Morrison, 1976) was used to examine patterns of ovarian follicles in different stages or conditions (primary, secondary, or tertiary stages of development). The statistical software package used to aid in the profile analysis was the SAS System for Windows© Release 7.00 (SAS Institute, Cary, North Carolina, 1998). The analysis was conducted on arcsine transformed proportions of follicles (parametric) and also on the ranks of these proportions (nonparametric). The arcsine transformation was executed to cause proportions to assume a nearly normal distribution (Zar, 1984). Because this transformation does not work well at the extreme ends of the range of possible values, for the proportions X/n (where $X=$ the number of follicles in a particular condition and $n=$ 50 follicles counted), the arcsine transformation was improved by replacing $0/n$ with $1/4n$ and n/n with $1-1/4n$, as suggested by Zar (Zar, 1984).

When plasma samples demonstrated levels of VTG less than the MDL of 0.267 μg VTG/mL, the value of one half of the MDL (0.134 μg VTG/mL) was used for the purposes of statistical analyses. Potential relationships between some endpoints were examined by calculating correlations between them. Pearson product-moment correlation coefficients and Spearman rank correlation coefficients were calculated with the aid of SYSTAT and SAS, respectively. Correlation coefficients were considered to be significant at $p < 0.05$ and highly significant $p < 0.01$.

Box plots were created with SYSTAT (SPSS Science, Chicago, 1998), and information in the box plots is described in the software help manual as follows (paraphrased). The center [horizontal] line marks the median of the sample. The length of each box shows the range within which the central 50% of the values fall, with the box edges (called hinges) at the first and third quartiles. Hspread is comparable to the interquartile range or midrange and is the absolute value of the difference between the values of the two hinges. Fences, which define outside and far outside values, are defined as follows:

Lower inner fence = lower hinge - (1.5 * Hspread)

Upper inner fence = upper hinge + (1.5 * Hspread)

Lower outer fence = lower hinge - (3 * Hspread)

Upper outer fence = upper hinge + (3 * Hspread)

The whiskers show the range of observed values that fall within the inner fences (within 1.5 Hspreads of the hinges). Because the whiskers extend to observed values and the fences need not correspond to observed values, the whiskers do not necessarily extend all the way to the inner fences. Asterisks mark values between the inner and outer fences, and circles mark far outside values, or those beyond the outer fences.

RESULTS

Exposure site water quality characteristics

Water quality parameters measured at each site were generally within tolerance guidelines for optimal health and growth of cyprinid fish (Table 3-2). (Billard, 1999a). Note that the first water quality measurements were taken just prior to deployment of the first fish cages. The dissolved oxygen reached supersaturation levels at sites LX and LW due to photosynthetic activity of phytoplankton, but the caged carp did not appear to suffer any obvious adverse effects. Water hardness at all sites reached levels greater than the optimum range for carp, but they can tolerate hardness exceeding 250 mg/L (Billard, 1999a). Hardness was greater at sites LX and LW than at the reference sites. There were consistent water temperature differences among sites; the temperature at LW was greatest, LX intermediate, and reference sites least. Average site temperatures ranged from 18.7 °C at LW to 15.7 °C at both reference sites. Conductivity measurements were

greatest at LW, less at LX, and least at WB and MC, indicating that carp caged at LW were receiving the greatest exposure and those caged at LX were receiving lesser exposure to Las Vegas Wash influent. Conductivity measurements at the reference sites were consistent with conductivity of main body lake water. Unionized ammonia and nitrite are not reported because they were not detected.

Exposure duration and fish survival

Although the goal was to expose fish at the different sites for the same length of time, unpredictable poor weather prevented timely retrieval of the cages such that the exposure duration varied from 38 to 49 d for pairs of cages (Table 3-3). Exposures began in late March or early April and lasted through mid-May. Four carp died during the exposure: two females caged at WB and two males caged at MC. Some fish were sexed incorrectly at the beginning of the study and placed into cages designated for the opposite sex. The fish that were sexed incorrectly were not included in subsequent analyses, except for calculations of initial and final mean weight per fish in each cage.

Condition

With the exception of male carp caged at LW and WB, the mean weight per fish in each cage was less at the end of the exposure than it was at

beginning (Table 3-4). Condition factors (K) among female carp ranged from 1.7e-1 to 2.9e-1. Median K for female carp ranged among sites from 2.2e-1 to 2.3e-1, and mean K for female carp caged at all sites was 2.3e-1 (Table 3-5). Mean or median K was not significantly different among female carp caged at any of the sites (Figure 3-2).

Condition factors for male carp ranged from 1.8e-1 to 4.0e-1. Median or mean K for male fish ranged among sites from 2.2e-1 to 2.4e-1 and from 2.2e-1 to 2.4e-1, respectively (Table 3-5). Among males, median K was significantly greater in carp caged at WB than in carp caged at LX (Figure 3-2). No other significant differences in median K were observed among males caged at different sites. Median K in male carp caged at LX and LW were not significantly different from those in males caged at both reference sites.

Gonadosomatic index

Among the female carp, GSI ranged from 4.6e-3 to 2.6e-1. Mean GSI for females ranged from 6.4e-2 to 1.2e-1 among sites (Table 3-5). Mean GSI for female carp caged at MC was significantly greater than that for females caged at LW (Figure 3-3). No other significant differences in mean GSI were detected among females caged at different sites. Mean GSI for females caged at LX and LW were not significantly different from those for females caged at both reference sites.

GSI ranged from 5.1e-3 to 7.8e-2 in the male carp. Mean GSI for males ranged among sites from 2.2e-2 to 4.2e-2 (Table 3-5). Male carp caged at the reference sites MC and WB demonstrated significantly greater mean GSI than males caged at LW and LX (Figure 3-3).

Histology of the hepatopancreas

Hepatopancreas samples from 58 male carp and 64 female carp were examined histologically (sample sizes reported in Table 3-6). One male carp caged at WB demonstrated periportal lymphatic infiltration in the hepatopancreas. Biliary stasis was observed in all but two carp subjected to histological examination of the hepatopancreas. One was an incorrectly sexed male caged with the female carp at MC. The other was a male caged at MC.

Hepatopancreas vacuolation

Hepatopancreas vacuolation scores observed in female carp ranged from the minimum of zero to the maximum of 3. Median hepatopancreas vacuolation scores for females ranged from 1 to 2 (Table 3-6). The median hepatopancreas vacuolation score observed for female carp caged at LX was significantly greater than scores for females at the other sites (Figure 3-4). No other significant differences in median hepatopancreas vacuolation score were observed among female carp caged at different sites.

Hepatopancreas vacuolation scores observed in male fish ranged from 1 to 2. The median hepatopancreas vacuolation score for males at each site was 1 (Table 3-6). Among the male carp caged at different sites, there were no significant differences in median hepatopancreas vacuolation scores (Figure 3-4).

Histology of the ovary

Ovary samples from 64 female carp (n = 16 from each site) were subjected to histological examination. In one female caged at reference site MC, a hardened growth on the ovary, darker in color than the rest of the tissue, was removed and preserved for histological examination. Examination revealed multiple hemorrhagic and proteinaceous cysts in the growth.

Ovarian follicle development

Profile analysis of arcsine transformed proportions of ovarian follicles in three stages of development (primary, secondary, and tertiary) revealed no significant differences in ovarian follicle development profiles among carp caged at the various sites (Figures 3-5 and 3-6). This finding indicates that female carp caged at all of the sites were in a similar state of ovarian development. When the same analysis was conducted on the ranks of the proportions (nonparametric analysis), the result was not different. Six female carp demonstrated no ovarian follicles developed beyond the primary

stage; two of these fish were found at each site but WB, where all of the fish examined had some ovarian follicles in the secondary or tertiary stages.

Ovarian follicle atresia

Atretic follicles were observed in the ovaries of 5 of the 64 female carp that were examined. One carp caged at MC demonstrated 1 atretic follicle, one caged at LW had 3 atretic follicles, one caged at WB had 4 atretic follicles, and two at LX had 2 and 1 atretic follicle per 50 follicles examined in each fish.

Histology of the testis

Testis samples from 63 male carp were examined histologically. Sample sizes were n = 15 for MC and n = 16 for LW, LX, and WB.

Spermatogenic activity

Only two of all male carp subjected to histological examination had spermatogenically inactive testes. Both of these fish were males that were placed into cages designated for females because they were incorrectly sexed due to their failure to produce milt and lack of discernable male secondary sex characteristics (more intense coloration, nuptial tubercles). All male carp that were subjected to histological examination of the testes and that were correctly sexed at the beginning of the exposure period

demonstrated spermatogenically active testes at the end of the exposure. Six males that were correctly sexed at the beginning of the study did not produce milt when pressure was applied to the abdomen at the end of the exposure period: three at MC, one at LW, one at WB, and one at LX.

Sertoli cell proliferation

Mild Sertoli cell proliferation, indicated by a low score of 1, was observed in 8 of the 63 male carp examined in this study. Seven of the fish that exhibited Sertoli cell proliferation were caged at site LX and one was caged at reference site WB. Male carp caged at LX demonstrated a significantly greater ($p < 0.01$) median Sertoli cell proliferation score than males caged at the other sites.

Plasma VTG

The inter-assay %CV for all samples analyzed in the VTG ELISA was 14.2 %. Plasma VTG concentrations in female carp ranged from less than the MDL (0.267 μg VTG/mL) to 17.0 mg/mL. Only one female carp, a fish with undeveloped ovaries caged at MC, did not demonstrate a measurable concentration of plasma VTG. The next smallest concentration of plasma VTG observed in a female was 20.4 μg VTG/mL, observed in a carp caged at LW. Median and mean plasma VTG concentrations for female carp ranged among sites from 5.33 to 6.84 mg VTG/mL and from 4.72 to 7.20 mg

VTG/mL, respectively (Table 3-7). Because the data were heteroscedastic and untransformed male VTG data were not normally distributed within each site, comparisons between plasma VTG concentrations in male and female fish were made using nonparametric statistics. There was no significant interaction of site and sex in the two-way ANOVA, so plasma VTG concentrations in male and female carp were analyzed separately for comparisons among sites within each sex. Female plasma VTG data were suitable for parametric analyses, which detected no significant differences in mean plasma VTG concentration among female carp caged at different sites (Figure 3-7). Nonparametric analyses did not give a different result.

Plasma VTG concentrations in male carp ranged from less than the MDL to 109 μg VTG/mL. The next greatest concentration measured in a male was 108 μg VTG/mL. All other male carp had plasma VTG concentrations less than 40 μg VTG/mL. Median and mean concentrations of plasma VTG for males ranged among sites from 1.19 to 4.34 μg VTG/mL and from 1.59 to 11.7 μg VTG/mL, respectively (Table 3-7). Plasma VTG data for male fish were not normally distributed and were heteroscedastic. Log_{10}-transformation of these data decreased the variances and normalized the distributions. Parametric analyses of log_{10}-transformed data and nonparametric analyses yielded the same results. The mean or median plasma VTG concentration observed in male carp caged at LX was

significantly greater than that in males caged at LW and WB (Figure 3-7). However, the mean or median plasma VTG concentrations in male carp caged at LW and LX were not significantly different from those observed in males caged at both reference sites.

Male and female carp caged at the same site differed significantly in median plasma VTG concentration within each site. The greatest plasma VTG concentration observed in a single male carp overlapped the single least concentration observed in a female within each site.

Plasma E2

Plasma E2 concentrations in female carp ranged from 152 to 965 pg E2/mL. Mean plasma E2 concentrations for females ranged from 340 to 493 pg E2/mL among sites (Table 3-8). Mean plasma E2 concentrations observed in female carp caged at MC and LX were significantly greater than those in females caged at WB and LW (Figure 3-8). Mean plasma E2 concentrations in female carp caged at LX and LW were not significantly different from those observed in females at both reference sites.

Plasma E2 concentrations observed in male carp ranged from 117 to 616 pg E2/mL. Mean plasma E2 concentrations in males ranged among sites from 265 to 449 pg E2/mL (Table 3-9). Mean plasma E2 concentrations in male

carp caged at LX were significantly greater than those in male carp caged at all other sites (Figure 3-8). The mean plasma E2 concentration in males caged at LW was not significantly different from those observed in male carp at either reference site.

Mean plasma E2 concentrations were significantly different between male and female carp caged at the same site only at the reference sites. At each site, the ranges of E2 concentrations observed in fish of different sexes overlapped substantially, but site means for females exceed those for males within each site.

Plasma T

Plasma T concentrations in female carp ranged from 208 to 1450 pg T/mL. Mean plasma T concentrations for females ranged among sites from 339 to 520 pg T/mL (Table 3-8). Median or mean plasma T concentration in female carp caged at MC was significantly greater than those observed in females caged at the other sites (Figure 3-9). Mean or median plasma T concentrations were not significantly different among female carp caged at the other sites. The mean or median plasma T concentrations observed in females caged at LX and LW were not significantly different from those observed in females caged at reference site WB. The results of parametric

statistical analysis on log_{10}-transformed data and nonparametric analysis were the same.

In the male carp, plasma T concentrations ranged from 155 to 1467 pg T/mL. Mean plasma T concentrations for male carp ranged among sites from 343 to 577 pg T/mL (Table 3-9). Mean or median plasma T concentrations were significantly greater in males caged at LX and LW than in males caged at WB. Mean or median plasma T concentrations observed in male carp caged at all sites did not differ significantly from those in males caged at reference site MC (Figure 3-9). Again, the results of statistical analyses were the same for parametric analysis of log_{10}-transformed data and nonparametric analysis.

Male and female carp caged at the same site differed significantly in mean or median plasma T concentration only at sites LW and LX; no differences in mean or median plasma T concentration were observed between the sexes within each reference site. The range of plasma T concentrations observed in females and males overlapped considerably within each site.

Ratio of E2 to T in blood plasma

Among female carp, E2:T ratio ranged from 0.32 to 2.71. Mean E2:T ratio ranged among sites from 1.00 to 1.42 (Table 3-8). Mean E2:T ratio

observed for female carp caged at LX was significantly greater than those for females caged at all other sites (Figure 3-10). No other site-to-site differences in E2:T ratio were observed among caged female carp.

E2:T ratio in caged male carp ranged from 0.21 to 1.51. Mean E2:T ratio in males ranged among sites from 0.67 to 0.88 (Table 3-9). No significant differences were observed in mean E2:T ratios among male fish caged at different sites (Figure 3-10).

Male and female carp caged at the same site differed significantly in mean E2:T ratio only at sites LX and LW. In all sites, there was a substantial overlap in the range of E2:T ratio values observed in male and female carp caged at the same site.

Plasma 11-KT

The distributions of plasma 11-KT concentrations in male and female carp did not meet the assumptions for parametric analysis, but results obtained with parametric analysis of $\log_{10}$-transformed data were the same as those obtained with nonparametric analysis. Among female carp, plasma 11-KT concentrations ranged from 65 to 765 pg 11-KT/mL. Mean plasma 11-KT concentrations in females ranged among sites from 199 to 455 pg 11-KT/mL (Table 3-8). Among female carp, the mean plasma 11-KT concentration of

fish caged at WB was significantly greater than those observed in female carp caged at the other sites (Figure 3-11). Mean plasma 11-KT concentration was not significantly different among females caged at the other sites.

In male carp, plasma 11-KT concentrations ranged from 65 to 1838 pg 11-KT/mL. Mean plasma 11-KT concentrations in males ranged from 320 to 810 pg 11-KT/mL among sites (Table 3-9). The mean plasma 11-KT concentration observed in male carp caged at LW was significantly less than those observed in male carp caged at the other sites (Figure 3-11). Mean plasma 11-KT concentrations were not significantly different among males caged at the remaining sites.

Male and female carp caged at the same site demonstrated a significant difference in mean plasma 11-KT concentration at sites LX and MC, but not at LW or WB. The ranges of plasma 11-KT concentrations observed in males and females caged at the same site overlapped at each site.

Ratio of E2 to 11-KT in blood plasma

E2:11-KT ratios in female carp ranged from 0.42 to 5.15 overall. Mean and median E2:11-KT ratios ranged among sites from 0.76 to 2.50 and from 0.76 to 2.45, respectively (Table 3-8). Plasma E2:11-KT ratios were not

normally distributed and were heteroscedastic. Log_{10}-transformation did not remedy these conditions completely, so nonparametric testing was used for both sexes. The median plasma E2:11-KT ratio in female carp caged at WB was significantly less than those in females caged at all other sites (Figure 3-12). Females caged at LW, LX, and MC did not differ significantly in median plasma E2:11-KT ratio.

Among male carp, plasma E2:11-KT ratios ranged from 0.21 to 5.04. Mean and median E2:11-KT ratios for males ranged among sites from 0.49 to 1.61 and from 0.46 to 1.21, respectively (Table 3-9). The median E2:11-KT ratio observed in male carp caged at LW was significantly greater than those for males at all other sites (Figure 3-12). The median E2:11-KT ratio for male carp caged at LX was not significantly different from those observed in males caged at either reference site.

Male and female carp caged at the same site differed significantly in median plasma E2:11-KT ratio at all sites but LW. The ranges of plasma E2:11-KT ratios observed in male and female carp caged at the same site overlapped within each site.

Correlations between biomarkers

Correlations between sex steroid hormones and ratios, other reproductive endpoints (GSI, VTG), and an indicator of health (condition factor K) were calculated in an attempt to identify relationships between biomarkers.

Females

Significant but weak ($r^2 < 0.20$) positive correlations exist between the following pairs of endpoints in female carp: K and VTG, E2 and T, E2 and VTG, E2:11-KT ratio and VTG (Table 3-10). A significant but weak ($r^2 < 0.10$) negative correlation exists between K and T. There is a significant positive correlation between GSI and K ($r^2 = 0.35$). Variation in both E2:T ratio and E2:11-KT ratio appear to be explained more by their significant negative relationships to the androgens than by their significant but weaker relationships to E2. The relationship between E2:11-KT ratio and 11-KT is the strongest observed for the endpoints examined in female carp in this study ($r^2 = 0.78$).

Males

Significant but weak ($r^2 \leq 0.20$) positive correlations were observed between the following pairs of endpoints examined in male carp: GSI and K, E2 and T, E2 and 11-KT, E2 and VTG, T and VTG, and 11-KT and VTG (Table 3-11). Significant but weak ($r^2 < 0.20$) negative correlations were demonstrated

between the following pair of endpoints: GSI and E2, GSI and E2:11-KT ratio, K and E2, and K and T. Variation in both E2:T ratio and E2:11-KT ratio appears to be explained more by their significant negative relationships to the androgens than to E2. There is no significant correlation between E2 and E2:11-KT ratio, and only a weak significant correlation between E2 and E2:T ratio. The relationship between 11-KT and E2:11-KT ratio is the strongest demonstrated for the endpoints examined in male carp ($r^2 = 0.80$), as it was for the females.

DISCUSSION AND CONCLUSIONS

Fish survival and condition

Carp mortalities during the field exposure were low in comparison with those from other fish caging studies conducted in sewage treatment plant effluents (Purdom et al., 1994) or in rivers receiving them (Nichols et al., 1999). The decrease in mean weight per fish in most cages might indicate that the carp were stressed simply by being held in cages. Considering the length of exposure (weeks), one would expect the fish to increase in weight rather than decrease. However, as explained previously, the methods used to measure the total weight of fish per cage at the time of cage placement and cage retrieval were different and might have introduced an error. In addition, the methods used to determine initial total weight were crude.

Condition factors were similar for female carp caged at the various sites. Male carp caged at WB were in better condition than those caged at LX, but there were no other significant differences among sites. Condition factors observed in this study are similar to those observed for male and female carp in our earlier similar study (Snyder et al., 2000a).

Histology of the hepatopancreas

One male carp caged at WB demonstrated periportal lymphatic infiltration in the hepatopancreas. This condition can be a response to parasite larval migration, protozoan infection, or mild viral or bacterial infection of the hepatopancreas. Because the condition was mild and localized, not associated with any known infectious agent in this fish, and found in only one fish at a reference site, it is not considered to be a significant finding. Because biliary stasis was observed in all but two carp subjected to histological examination of the hepatopancreas, the lesion does not appear to be associated with exposure to the influent of the Las Vegas Wash. Among male carp caged at different sites, there was no significant difference in hepatopancreas vacuolation score. Female carp caged at LX demonstrated a greater degree of hepatopancreas vacuolation than female carp caged at any other site. This finding may or may not be related to exposure to Las Vegas Wash influent.

Gonadal development and histology

Previous studies have demonstrated decreased GSI in male, female, and intersex fish exposed to sewage effluent (Harries et al., 1997; Jobling et al., 1998). GSI was examined in carp caged at Lake Mead to determine whether exposure to the Las Vegas Wash influent reduced gonadal growth and to determine the state of sexual development of the fish to aid in interpretation of any site-to-site differences in sex steroid hormone concentrations measured in the fish. Female carp caged at LW demonstrated reduced ovarian growth (as GSI) relative to females caged at MC, but no other significant differences were observed among females. There also were no significant differences in follicle development profile among female carp caged at different sites, indicating that the female carp all were in a similar state of reproductive development.

Mean GSI in male carp caged at LW and LX was reduced relative to those observed in males caged at the reference sites, indicating that exposure to the Las Vegas Wash influent caused a reduction in testicular growth relative to controls. Alternatively, it is possible that male carp had begun to spawn at LW and LX and reduced their testicular weight by shedding milt. No obvious evidence of recent spawning (bloodshot, flaccid testes) was observed in any of the male carp used in this study, but this "spent" appearance lasts only a few days (Boon Swee et al., 1966). LW and LX

reached temperatures great enough for carp to spawn (Boon Swee et al., 1966; Manning et al., 1984; Horvath, 1986), although the temperatures probably were not in the optimal spawning temperature range for carp in this geographic region. Spawning behavior in male carp is controlled by the availability of females ready to spawn. We had hoped to avoid the influence of one sex on the reproductive endocrinology of the other by caging them separately. Four female carp were sexed incorrectly at the beginning of the study and placed in the cage designated for males at LX, and at least one of those was a female with developing ovaries. One vitellogenic female carp was caged with the males at LW. If any of the female carp caged with the males were ready to spawn, they might have released pheromones that could cause the male carp to come into full spawning condition. Male carp in the cages might have been exposed to wild female carp attempting to spawn, but spawning behavior of carp involves the male chasing the female and the two (or more) fish thrashing around a spawning substrate together (Panek, 1987). It would not be possible for caged male carp to chase a female outside the cage. It is not known whether male carp would be capable of taking part in spawning with a wild female through the cage mesh, although the fish might consider the mesh a suitable substrate on which to scatter eggs (Billard, 1999b). It seems more likely that testicular growth was inhibited in male carp exposed to Las Vegas Wash influent.

The issue of female carp inadvertently caged with males at sites where water temperatures approach those appropriate for spawning might be important in interpretation of steroid hormone profile alterations (below) as well. In the goldfish (*Carassius auratus*), a species closely related to carp, a pheromone secreted by ovulatory females induces a gonadotropin surge in the males, (Kobayashi et al., 1986; Dulka et al., 1987; Sorensen et al., 1988; Van Der Kraak et al., 1989) thereby altering the steroid hormone profiles of males to coordinate spermiation in males with ovulation in females. Others have hypothesized that the same pheromonal cues are utilized by carp (Barry et al., 1990).

On the basis of GSI observed by other researchers in common carp or mirror carp in different states of sexual development, both male and female carp used in this study appeared to be in the mid- to late stages of gonadal recrudescence at the end of the exposure period (Parameswaran et al., 1972; Shikhshabekov, 1972; Gupta, 1975; Crivelli, 1981; Manning et al., 1984; Horvath, 1986; Guha et al., 1987; Tyler et al., 1990). The intermediate stage of recrudescence is the optimum time period for detection of alterations in the endpoints examined in this study (McMaster et al., 1992)

Interestingly, of eight male carp in this study that demonstrated Sertoli cell proliferation, 7 were caged at LX. One was caged at WB. The median Sertoli cell proliferation score was greater in male carp caged at LX than in males caged at any other site. Although the lesions all were mild (score of 1), they might indicate that male carp caged at LX were exposed to an exogenous estrogenic chemical (Miles-Richardson et al., 1999a; Miles-Richardson et al., 1999b). However, the mechanism resulting in Sertoli cell proliferation is not known, and the effect might not be mediated through the estrogen receptor. All male carp that were correctly sexed at the initiation of the exposure period demonstrated spermatogenically active testes. Six of the male carp caged in the field did not produce milt at the end of the exposure period, but there was no apparent relationship between exposure site and failure of male carp to produce milt.

The ovary of one female carp caged at reference site MC contained a growth characterized by multiple hemorrhagic and proteinaceous cysts. This condition can result from septicemia, protozoan infestation of the gonad (not seen here), or as a spontaneous idiopathic condition (S. Fitzgerald, personal communication). Because this lesion was observed in only one carp and because the fish was caged at a reference site, it is unlikely to be a result of any toxic effect arising during the exposure period. Incidence and severity of ovarian follicle atresia and incidence of female carp demonstrating ovaries

with no follicle development beyond the primary stage did not appear to be related to exposure sites.

Plasma VTG

There were no significant differences in plasma VTG concentration among female carp caged at different sites, although the median plasma VTG concentration in females caged at LX appears to be slightly elevated relative to those in females caged at the other sites. Plasma VTG concentrations appear to be elevated slightly in male carp caged at LX as well, but the median plasma VTG concentration is not significantly different from that observed for male carp caged at MC. The patterns observed for median plasma VTG concentrations among sites were similar for male and female carp and appeared to parallel patterns of median plasma E2 concentrations among sites. This observation indicated that any differences (non-significant in this case) in plasma VTG concentrations among carp caged at different sites were related to changes in endogenous plasma E2 concentrations. Environmental contaminants or drugs might cause VTG induction in fish by binding to the estrogen receptor (ER) directly (Jobling et al., 1993) or by indirectly causing an increase in endogenous plasma E2 concentration (Janssen et al., 1997; Giesy et al., 2000). In the latter case, the contaminant might increase synthesis of E2, for example, by positive feedback at various points in the hypothalamic-pituitary-gonad axis or by

altering the activity of the enzyme aromatase, which converts T to E2. Alternatively a contaminant might decrease metabolism and clearance of E2 from the body, thereby increasing endogenous circulating levels of E2 (Janssen et al., 1997).

Plasma sex steroid hormone concentrations

With regard to plasma steroid hormone concentrations, we were concerned primarily with determining whether the influent of the Las Vegas Wash at LW or LX significantly affected estrogen to androgen (E:A) ratios (E2:T ratio or E2:11-KT ratio) in the caged carp in a manner that could not be explained by site physical characteristics. E2:T ratio was greater in female carp caged at LX than in females caged at the other sites. This difference seems to be related to elevated plasma E2 concentrations in female carp caged at LX, although the plasma E2 concentrations observed in female carp caged at LX were not significantly different from those observed in females caged at MC. Male carp caged at LW appeared to have a reduced E2:T ratio (due to a non-significant increase in plasma T) relative to male carp caged at the other sites, but this difference was not statistically significant.

E2:11-KT ratios in female carp caged at WB were significantly reduced in comparison to those observed in female fish caged at the other sites. The increase was due to an elevation in plasma 11-KT concentration observed in

female carp caged at WB. The reason for this difference is not known. Average water temperature at WB was the same as was observed at MC, and condition and GSI do not seem to be related to the difference in E2:11-KT ratio. Similarly, in our previous study, female carp caged at WB demonstrated a relatively lesser plasma E2:11-KT ratio than females caged at other sites.

E2:11-KT ratio was elevated in male carp caged at LW relative to those observed in males caged at other sites. This appears to be due to a decrease in plasma 11-KT concentration. USGS reported decreased plasma 11-KT and plasma E2 concentrations and increased plasma VTG in wild male carp collected from Las Vegas Bay in May 1995. In the present study, the carp were retrieved from the cages in May as well, and male carp caged at LW, but not LX, demonstrated decreased plasma 11-KT. However, the caged male carp did not exhibit decreased plasma E2 concentration at either LW or LX. Instead, male carp caged at LX had elevated plasma E2 concentrations relative to those observed in males caged at other sites. Also, although there might have been a slight (but non-significant) increase in plasma VTG concentration in male carp caged at LX, this increase was accompanied by an increase in plasma E2.

Because water temperature at LW and possibly LX were great enough for spawning in carp, it is possible that the observed alterations in E:A ratios in male carp caged at LW were due to water temperature. Dramatic fluctuations in plasma sex steroid levels, particularly 11-KT, have been documented in male carp just prior to spawning and coincident with temperature increase from 16 °C to 24 °C, which induces spawning in carp (Barry et al., 1990). As discussed previously, female carp were inadvertently place in cages designated for male carp, and the presence of females at appropriate spawning temperatures might cause alterations in male carp steroid hormone profiles.

E2:T ratio was elevated in female carp caged at LX but not in females caged at LW. As with male carp, dramatic fluctuations in plasma sex steroid concentrations occur just prior to ovulation and spawning and coincident with temperature increase from 16 °C to 24 °C, which induces ovulation in female carp (Santos et al., 1986). These changes might include an increase in circulating plasma E2, but a coincident and more drastic increase in T might be expected based on previous reports of steroid hormone profiles in female carp during ovulation (Santos et al., 1986; Aida, 1988). No increase in plasma T was observed in female carp caged at LX or LW. However, hormone profiles previously reported for female carp were measured in fish subjected to a water temperature increase from 16 °C to 24 °C in a very

short period of time (Santos et al., 1986). If this temperature increase took place more gradually, as might be expected in the environment, the fluctuations in circulating plasma sex steroid levels might have been somewhat different.

Steroid hormone concentrations overall were rather low but similar to concentrations reported previously for adult male and female carp held at 18 to 20 °C (Yano et al., 1986) or for wild-caught carp undergoing gonadal recrudescence (Goodbred et al., 1997). The low steroid hormone concentrations observed in carp caged at the field sites might be representative of typical inter-spawning concentrations or might indicate that the fish at all sites were experiencing stress that prevented their sex steroid concentrations from increasing. Stress is known to cause significant reductions in circulating sex steroid concentrations in some species of fish (McMaster et al., 1992), possibly including carp (Santos et al., 1986). The failure of the carp to gain weight over the course of the study lends support to the hypothesis that stress prevented sex steroid cycling. However, the similarity of circulating plasma sex steroid concentrations observed in the caged carp to concentrations reported previously in unstressed fish indicated that these were normal levels for carp between spawning periods.

Correlations

The observed correlation between GSI and K in female carp is not surprising, since it is well-known that female fish in good condition will invest more energy in egg production than those in poor condition. E:A ratios in both sexes appear to be dominated by androgen levels. Given the greater importance of androgens as sex hormones relative to estrogens in male fish, the dominance of androgens in the E:A ratios for males also is not surprising. The dominance of androgens in the E:A ratios in females might indicate a later rather than middle stage of gonadal recrudescence for female carp at the end of the exposure period. E2 levels tend to be greater in female fish during the middle stages of ovarian recrudescence to facilitate vitellogenesis (Aida, 1988). Later in recrudescence when ovarian growth (which is controlled primarily by VTG uptake into oocytes (Tyler et al., 1996)) approaches its maximum, estrogen levels decline and T levels increase in female fish. The increase in T might be involved in inducing final oocyte maturation in preparation for spawning (Aida, 1988).

In conclusion, the results of the current study are somewhat different from the results of our previous similar study (Snyder et al., 2000a). Significant VTG induction observed in male carp caged at LW in the earlier study was not observed male carp caged at LW in the current study. However, the hypothesis that the mild (non-significant) Sertoli cell proliferation (one male

carp caged at LX and one at LW) observed in male carp exposed to Las Vegas Wash influent in the earlier study was related to site was supported by the finding of increased Sertoli cell proliferation in the testes of male carp caged at LX in the current study. It cannot be concluded at this time that Sertoli cell proliferation is caused exclusively by exposure to estrogenic substances, though, and the lesions observed were mild. Similar to the results of the first study, in this study there were no observed effects on plasma steroid hormone profiles related to the Las Vegas Wash influent that do not have potential explanations rooted in site physical characteristics or differences in reproductive timing. Most importantly, it appears that the Las Vegas Wash influent might have inhibited testicular growth in male carp caged at LW and LX and decreased plasma 11-KT concentrations in male carp caged at LW. The finding of decreased plasma 11-KT concentration in male carp exposed to Las Vegas Wash influent is consistent with effects reported in wild male carp caught in Las Vegas Bay by USGS researchers. However, because water temperatures at the LW and LX sites were great enough to induce spawning in carp in the current study, the current findings of reduced GSI and plasma 11-KT concentrations are stated tentatively.

As discussed in our earlier study, the next logical step in attempting to address our original objective is to compare concentrations of estrogenic contaminants measured in the Las Vegas Wash and Las Vegas Bay to

responses observed in the caged carp to determine which chemical contaminants might be present at concentrations great enough to produce the observed effects. In addition, given the somewhat questionable or marginal responses observed in the carp, it would be prudent to attempt to cage a species more sensitive to exposure to estrogenic chemicals, such as rainbow trout, in the Las Vegas Wash and Las Vegas Bay, to determine more conclusively whether contaminants that are estrogenic to fish are present in the influent. Trout were not considered for use in our first two fish caging trials because of the great likelihood that the trout could not tolerate the water quality conditions in the Las Vegas Wash. However, a short-term exposure of trout to the influent during the colder winter months might prove successful.

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Table 3-1. Descriptions of different ovarian follicle types identified in common carp ovaries during histological examination. Taken from (Miles-Richardson et al., 1999).

Follicle Type	Description
Primary	abundant basophilic cytoplasm (deep blue color), large pale central nucleus, no yolk vesicles
Secondary	eosinophilic yolk vesicles appear in vacuolated cytoplasm, granulosa cells surround central oocyte
Tertiary	largest follicle type, numerous eosinophilic yolk globules fill cytoplasm
Atretic	degenerative follicle with collapsed, irregular contour; macrophages invading follicle

Table 3-2. Water quality parameters measured at caged carp exposure sites in Lake Mead, Nevada. Temp. = temperature; DO = dissolved oxygen; Cond. = conductivity; Turb. = turbidity, in national turbidity units. [a] Measured as $CaCO_3$. [b] Total ammonia.

Site: LX

Date	Temp. (° C)	pH	DO (mg/L)	Cond. (μS)	Turb. (NTU)	Alkalinity (mg/L)	Hardness[a] (mg/L)	Ammonia[b] (mg/L)
03.26	16.9	8.61	12.83	1088	0.8			
04.16	16.5	8.10	9.87	1492	*	111	430	0.0
04.26	17.2	8.05	9.62	1099	*	116	322	0.0
05.05	17.6	7.97	9.74	1246	*	116	390	0.0
Average	17.1	8.18	10.52	1231	0.8	114	381	0.0

Site: MC

Date	Temp. (° C)	pH	DO (mg/L)	Cond. (μS)	Turb. (NTU)	Alkalinity (mg/L)	Hardness[a] (mg/L)	Ammonia[b] (mg/L)
03.26	14.2	8.23	10.13	894	0.0			
04.16	14.1	7.89	9.81	878	*	108	292	0.0
04.23	15.9	8.02	9.70	*	*	112	298	0.0
04.26	16.5	8.05	9.84	889	*	116	286	0.0
05.05	16.3	8.09	9.98	872	*	112	270	0.0
Average	15.7	8.01	9.83	880	0.0	112	287	0.0

Table 3-2. (Continued). Water quality parameters measured at caged carp exposure sites in Lake Mead, Nevada. Temp. = temperature; DO = dissolved oxygen; Cond. = conductivity; Turb. = turbidity, in national turbidity units. [a] Measured as $CaCO_3$. [b] Total ammonia.

Site: WB

Date	Temp. (° C)	pH	DO (mg/L)	Cond. (μS)	Turb. (NTU)	Alkalinity (mg/L)	Hardness[a] (mg/L)	Ammonia[b] (mg/L)
03.26	14.9	8.00	9.83	883	0.4			
04.16	14.4	8.06	9.83	874	*	109	294	0.0
04.23	15.3	7.83	9.62	*	*	110	296	0.0
04.26	16.1	7.88	9.93	877	*	115	274	0.0
05.05	17.0	7.88	9.67	861	*	113	274	0.0
Average	15.7	7.91	9.76	871	0.0	112	285	0.0

Site: LW

Date	Temp. (° C)	pH	DO (mg/L)	Cond. (μS)	Turb. (NTU)	Alkalinity (mg/L)	Hardness[a] (mg/L)	Ammonia[b] (mg/L)
03.26	18.7	8.53	13.17	1636	4.1			
04.16	18.3	8.07	9.36	2070	*	105	574	0.0
04.26	20.0	7.87	7.91	1911	*	116	592	0.0
05.05	17.9	8.10	9.80	*	*	116	400	0.0
Average	18.7	8.14	10.06	1872	0.0	112	522	0.0

Table 3-3. Fish cage placement and retrieval dates, exposure duration, mortalities, and other general data.

Cage (site/sex)	Placement Date	Retrieval Date	Exposure Duration (d)[a]	Initial Number	Final Number	Mortalities	Number Sexed Incorrectly[c]
LW male	04.05	05.13	38	30	30	0	1
LW female	04.05	05.13	38	30	30	0	4
WB male	03.29	05.17	49	30	30	0	4
WB female	03.29	05.17	49	30	28	2	0
MC male	04.01	05.11	40	30	28	2	3
MC female	04.01	05.11	40	30	30	0	2
LX male	04.07	05.19	42	31[b]	31	0	4
LX female	04.07	05.19	42	30	30	0	2

[a] including day of placement, but not day of retrieval

[b] one extra male inadvertently added to cage

[c] number of fish sexed incorrectly and placed into a cage designated for the opposite sex

NA = not applicable

Table 3-4. Weights of carp at initiation and completion of the field exposure period.

CAGE (site/sex)	INITIAL Total Wt. (kg)	INITIAL Mean Wt. per Fish (g)[a]	FINAL Total Wt. (kg)	FINAL Mean Wt. per Fish (g)[b]
LW male	4.68	156	4.841	161
LW female	4.68	156	4.515	151
WB male	3.67	122	3.742	125
WB female	3.67	122	3.344	119
MC male	4.02	134	3.503	125
MC female	4.78	159	4.372	146
LX male	4.08	132	4.024	130
LX female	4.26	142	4.010	134

[a] initial total weight (kg) divided by 30 fish (number initially weighed, 31 fish for site LX); converted to grams

[b] final total weight (kg) divided by number of fish when cage was retrieved; converted to grams

Table 3-5. Length, weight, gonadosomatic index (GSI), and condition factor (K) data for adult male and female common carp caged at Lake Mead. Means are reported as mean ± 1 SEM.

Site	MALES			FEMALES		
	n	mean	range	n	mean	range
MC						
Length (cm)	25	17.6 ± 0.5	14.3 - 23.8	28	18.6 ± 0.3	15.3 - 21.9
Weight (g)	25	130 ± 11	59 - 298	28	149 ± 7	80 - 240
GSI	16	3.9e-2 ± 3.5e-3	1.7e-2 - 6.2e-2	16	1.2e-1 ± 1.8e-2	4.6e-3 - 2.6e-1
K	25	2.3e-1 ± 7.6e-3	1.9e-1 - 4.0e-1	28	2.3e-1 ± 3.0e-3	1.9e-1 - 2.5e-1
LW						
Length (cm)	29	19.0 ± 0.4	16.4 - 24.1	26	18.6 ± 0.4	14.6 - 22.6
Weight (g)	29	161 ± 9	111 - 311	26	152 ± 9	63 - 232
GSI	16	2.5e-2 ± 3.3e-3	5.1e-3 - 5.1e-2	16	6.4e-2 ± 1.2e-2	4.9e-3 - 1.5e-1
K	29	2.3e-1 ± 4.2e-3	1.8e-1 - 2.8e-1	26	2.3e-1 ± 4.7e-3	1.9e-1 - 2.9e-1
WB						
Length (cm)	26	17.1 ± 0.4	14.2 - 23.1	28	17.2 ± 0.3	14.7 - 21.0
Weight (g)	26	126 ± 10	73 - 296	28	119 ± 5	79 - 208
GSI	16	4.2e-2 ± 3.6e-3	2.5e-2 - 7.8e-2	16	9.9e-2 ± 1.4e-2	2.1e-2 - 2.0e-1
K	26	2.4e-1 ± 3.9e-3	2.0e-1 - 2.8e-1	28	2.3e-1 ± 4.1e-3	1.7e-1 - 2.7e-1

Table 3-5. (Continued). Length, weight, gonadosomatic index (GSI), and condition factor (K) data for adult male and female common carp caged at Lake Mead. Means are reported as mean ± 1 SEM.

Site	MALES			FEMALES		
	n	mean	range	n	mean	range
LX						
Length (cm)	26	17.8 ± 0.5	13.1 - 22.4	28	18.0 ± 0.3	15.1 - 23.1
Weight (g)	26	129 ± 9	79 - 235	28	133 ± 7	90 - 265
GSI	16	2.2e-2 ± 2.6e-3	5.2e-3 - 4.1e-2	16	8.7e-2 ± 1.4e-2	4.8e-3 - 1.6e-1
K	26	2.2e-1 ± 6.9e-3	1.8e-1 - 3.6e-1	28	2.3e-1 ± 4.0e-3	1.9e-1 - 2.7e-1

Table 3-6. Scores for hepatocellular vacuolation of the hepatopancreas for male and female carp, reported as the site median score with minimum and maximum values in parentheses. Vacuolation was scored on a scale as follows: 0 = no vacuolation, 1 = mild vacuolation with small vacuoles spread throughout the cytoplasm, 2 = moderate vacuolation with larger coalescing vacuoles appearing as large clear zones in many hepatocytes, 3 = severe vacuolation where all or most of the cytoplasm has lost its normal pink coloration due to confluent, large, clear vacuoles.

	MALES			FEMALES		
SITE	n	Score	Range	n	Score	Range
MC	14	1	(1 - 2)	16	1	(1 - 2)
LW	16	1	(1 - 2)	16	1	(0 - 2)
WB	14	1	(1 - 2)	16	1	(0 - 2)
LX	14	1	(1 - 2)	16	2	(1 - 3)

Table 3-7. Concentrations of vitellogenin (VTG) in blood plasma of adult male (μg/mL) and female (mg/mL) carp. Site medians are reported with minimum and maximum values in parentheses. Note that male plasma VTG concentrations are reported in micrograms per milliliter and female plasma VTG concentrations in milligrams per milliliter. ND = not detected (< 0.267 μg/mL).

	MALES		FEMALES	
SITE	n	VTG (μg/mL)	n	VTG (mg/mL)
MC	25	1.93 (ND - 26.3)	28	5.73 (ND - 13.5)
LW	29	1.19 (ND - 5.73)	25	5.82 (0.020 - 15.6)
WB	26	1.26 (ND - 109)	28	5.33 (0.060 - 11.0)
LX	26	4.34 (ND - 108)	28	6.84 (0.034 - 17.0)

Table 3-8. Concentrations of E2, T, and 11-KT, and ratios of E2:T and E2:11-KT in blood plasma of adult female carp. Site medians are reported with minimum and maximum values in parentheses. E2 = 17β-estradiol, T = testosterone, 11-KT = 11-ketotestosterone.

		FEMALES				
SITE	n	E2 (pg/mL)	T (pg/mL)	E2:T	11-KT (pg/mL)	E2:11-KT
MC	28	457 ± 24 (279 - 774)	520 ± 54 (302 - 1450)	1.00 ± 0.07 (0.32 - 1.84)	199 ± 14 (66 - 388)	2.50 ± 0.15 (1.47 - 4.72)
LW	25	348 ± 15 (243 - 505)	345 ± 28 (208 - 770)	1.09 ± 0.06 (0.42 - 1.64)	263 ± 41 (65 - 762)	2.22 ± 0.30 (0.42 - 5.15)
WB	28	340 ± 15 (152 - 477)	339 ± 20 (217 - 751)	1.06 ± 0.06 (0.47 - 1.65)	455 ± 18 (301 - 765)	0.76 ± 0.04 (0.43 - 1.13)
LX	28	493 ± 31 (214 - 965)	371 ± 24 (216 - 715)	1.42 ± 0.11 (0.71 - 2.71)	236 ± 16 (84 - 387)	2.28 ± 0.17 (0.84 - 4.48)

Table 3-9. Concentrations of E2, T, and 11-KT, and ratios of E2:T and E2:11-KT in blood plasma of adult male carp. Site medians are reported with minimum and maximum values in parentheses. E2 = 17β-estradiol, T = testosterone, 11-KT = 11-ketotestosterone.

		MALES				
SITE	n	E2 (pg/mL)	T (pg/mL)	E2:T	11-KT (pg/mL)	E2:11-KT
MC	25	343 ±15 (228 - 533)	426 ± 34 (241 - 818)	0.88 ± 0.05 (0.48 - 1.51)	588 ± 72 (109 - 1372)	0.99 ± 0.16 (0.24 - 3.36)
LW	29	323± 16 (191 - 564)	577 ± 50 (274 - 1467)	0.67 ± 0.06 (0.21 - 1.43)	320 ± 51 (65 - 1140)	1.61 ± 0.21 (0.27 - 5.04)
WB	26	265 ± 15 (117 - 409)	343 ± 32 (155 - 819)	0.85 ± 0.05 (0.37 - 1.42)	615 ± 58 (204 - 1655)	0.49 ± 0.04 (0.21 - 0.97)
LX	26	449 ± 22 (178 - 616)	577 ± 46 (262 - 1196)	0.86 ± 0.06 (0.46 - 1.37)	810 ± 90 (217 - 1838)	0.75 ± 0.09 (0.26 - 1.69)

Table 3-10. Correlation coefficents (r) between plasma sex steroid concentrations and ratios, gonadosomatic index (GSI), condition factor (K), and plasma vitellogenin (VTG) in adult female carp. Correlation coefficients are Spearman rank correlation coefficients unless otherwise noted. E2 = 17β-estradiol, T = testosterone, 11-KT = 11-ketotestosterone.

Endpoint	GSI	K	E2	T	11-KT	E2:T	E2:11-KT	VTG
GSI	1.000							
K	0.592** [a]	1.000						
E2	0.051 [a]	-0.163 [a]	1.000					
T	0.026	-0.206*	0.435**	1.000				
11-KT	-0.011	0.004	-0.085	-0.122	1.000			
E2:T	-0.035	-0.008	0.425**	-0.556**	-0.004	1.000		
E2:11-KT	0.065	-0.044	0.478**	0.236*	-0.886**	0.232*	1.000	
VTG	0.136 [a]	0.248** [a]	0.378** [a]	0.129	-0.174	0.173	0.326**	1.000

[a] Pearson product-moment correlation coefficient
* 0.01 < or = p < 0.05 for correlation coefficient
** p < 0.01 for correlation coefficient

Table 3-11. Correlation coefficents (r) between plasma sex steroid concentrations and ratios, gonadosomatic index (GSI), condition factor (K), and plasma vitellogenin (VTG) in adult male carp. Correlation coefficients are Spearman rank correlation coefficients unless otherwise noted. E2 = 17β-estradiol, T = testosterone, 11-KT = 11-ketotestosterone.

Endpoint	GSI	K	E2	T	11-KT	E2:T	E2:11-KT	VTG
GSI	1.000							
K	0.339**	1.000						
E2	-0.432**	-0.337**	1.000					
T	-0.075	-0.214*	0.443**	1.000				
11-KT	0.108	-0.145	0.288**	-0.027	1.000			
E2:T	-0.193 [a]	-0.063	0.305**	-0.670**	0.160	1.000		
E2:11-KT	-0.286*	-0.006	0.129	0.240*	-0.895**	-0.040	1.000	
VTG	0.178	-0.119	0.202*	0.246*	0.217*	-0.111	-0.109	1.000

[a] Pearson product-moment correlation coefficient

* 0.01 < or = p < 0.05 for correlation coefficient

** p < 0.01 for correlation coefficient

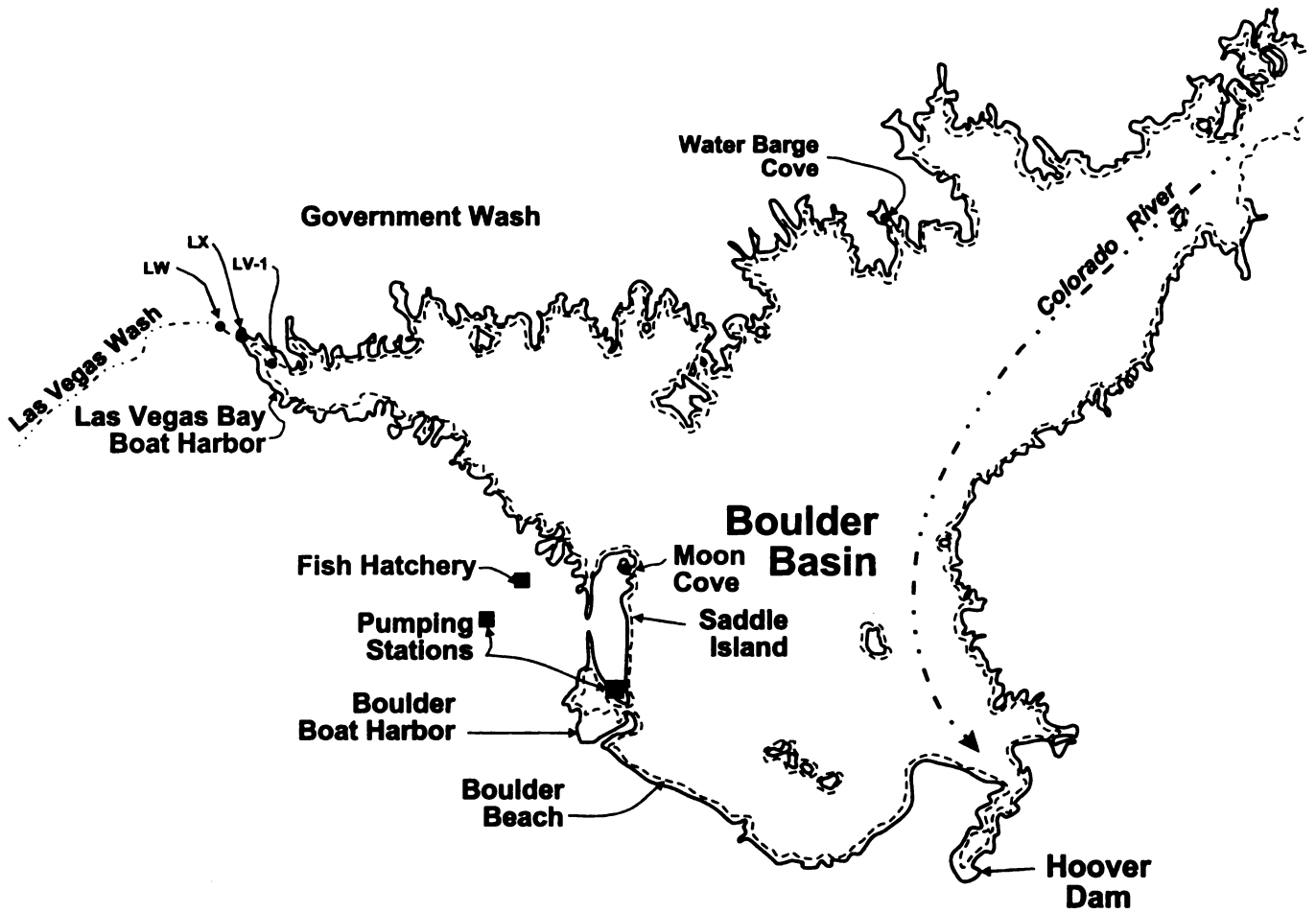

Figure 3-1. Map of the Boulder Basin in Lake Mead, Nevada. Sites of interest in this study are the Fish Hatchery (HF), Moon Cove (MC), Water Barge Cove (WB), Las Vegas Wash (LW), and Las Vegas Bay (LX).

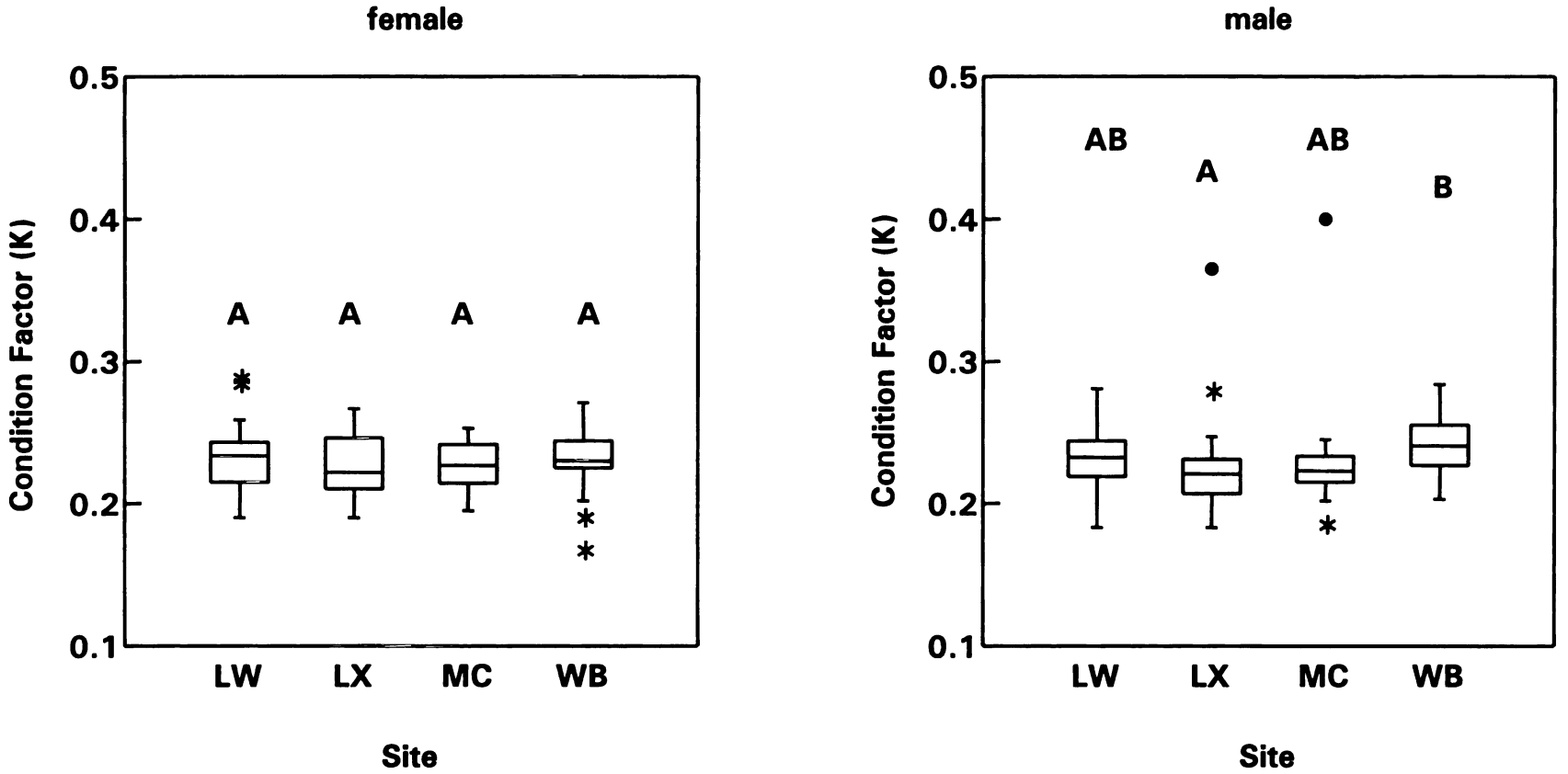

Figure 3-2. Condition factor (K), calculated as $K = W/L^3 \times 10{,}000$ where W = weight in g and L = standard length in mm. Letters above boxes represent Tukey-like groupings within each sex. The center horizontal lines mark the medians for each site, and upper and lower edges of the boxes (hinges) represent first and third quartiles. Whiskers show the range of observed values that fall within the inner fences. Asterisks mark "outside values" (those between the inner and outer fences), and circles mark "far outside values" (those beyond the outer fences). See text under **"Statistical analyses"** for discussion of terms.

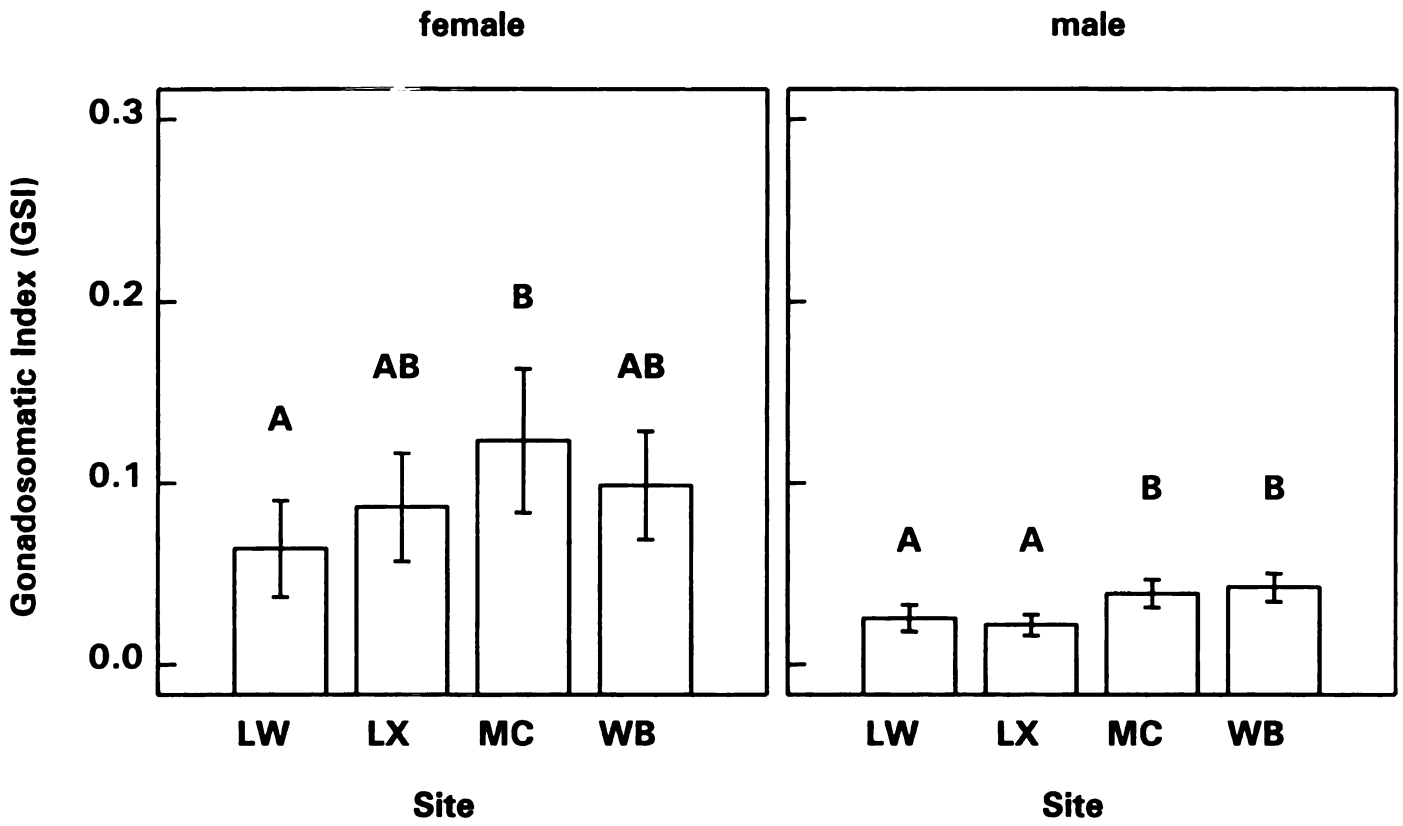

Figure 3-3. Gonadosomatic index (GSI). Bar heights represent means, and error bars are 2 SEM. Letters above the bars represent Tukey groupings within each sex.

$$GSI = \frac{\text{gonad weight}}{(\text{body weight - gonad weight})}$$ where all weights are in grams.

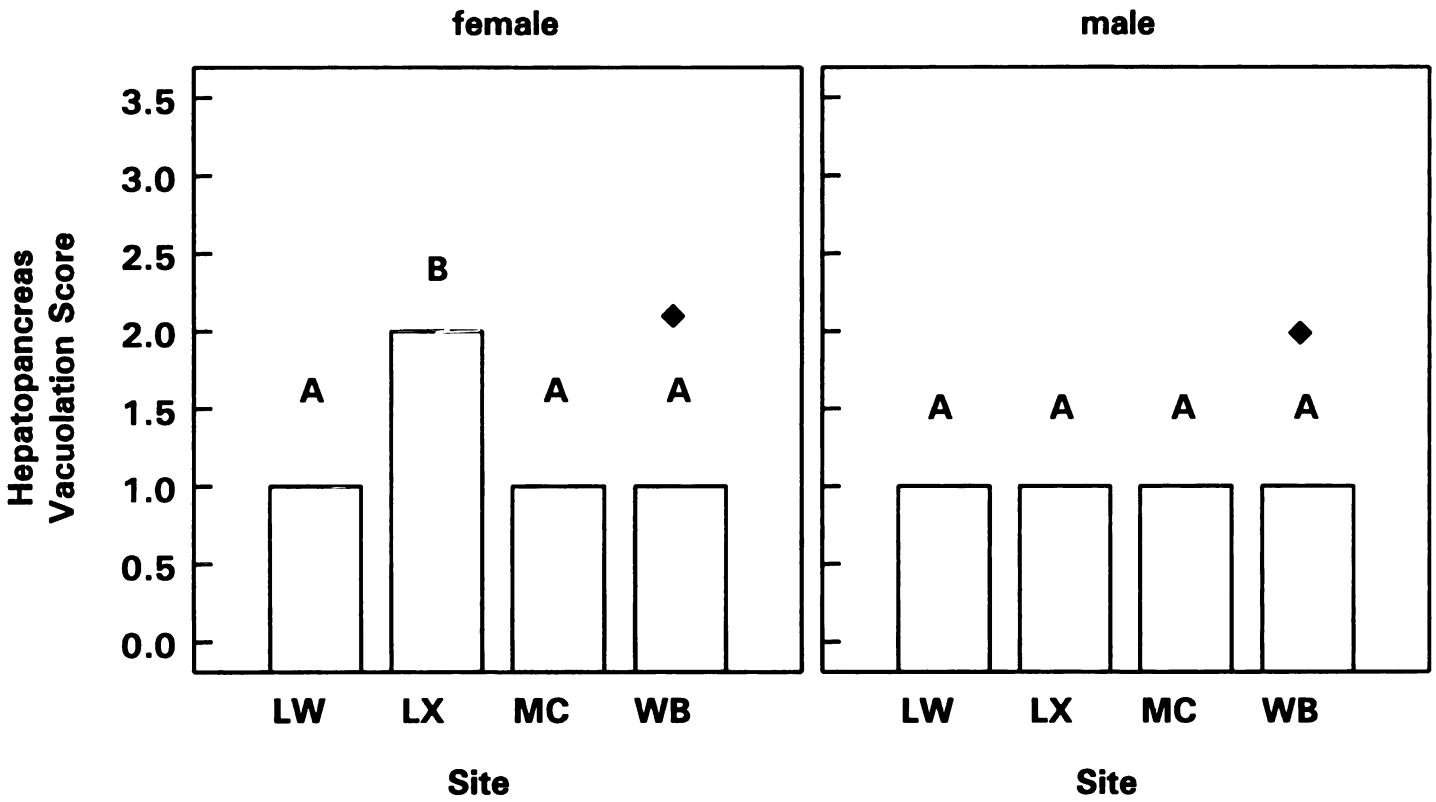

Figure 3-4. Vacuolation of the hepatopancreas in adult female and male carp. Bar height represents median vacuolation score for each site. Letters above the bars represent Tukey groupings within each sex. ♦ = significant difference in median vacuolation score from fish of the opposite sex caged at the same site.

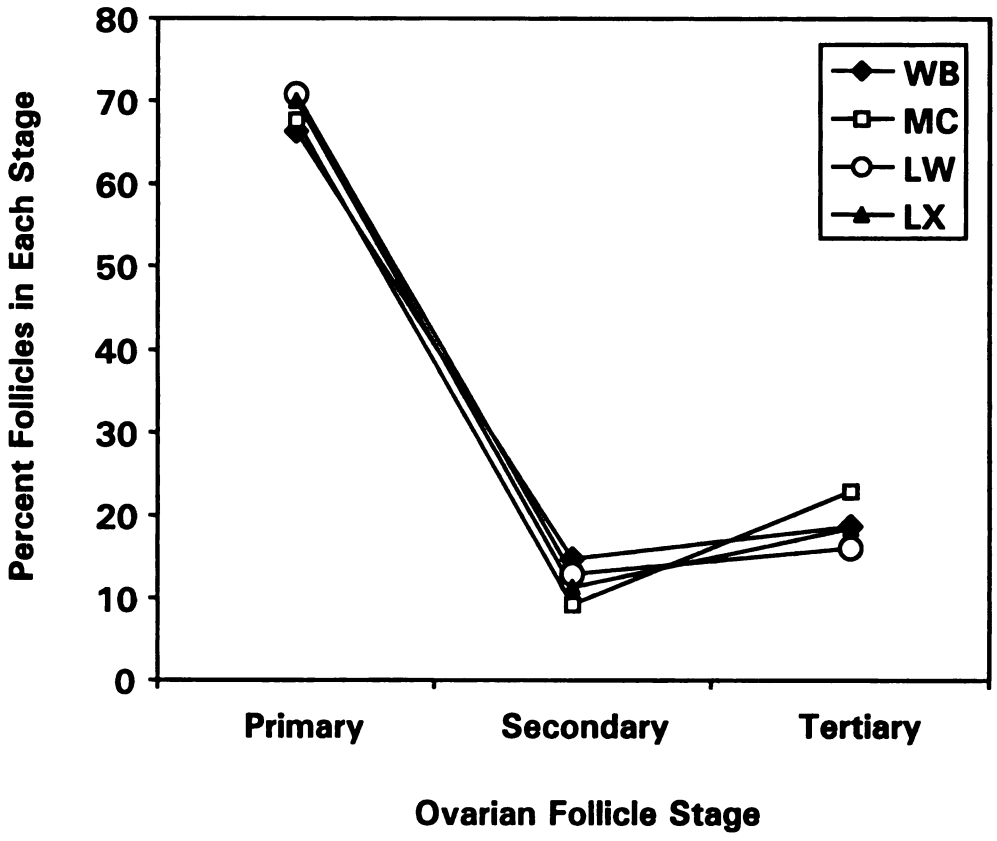

Figure 3-5. Profiles of percentages of ovarian follicles in each of three stages: primary, secondary, and tertiary. Each point represents the mean percentage of follicles in the corresponding stage.

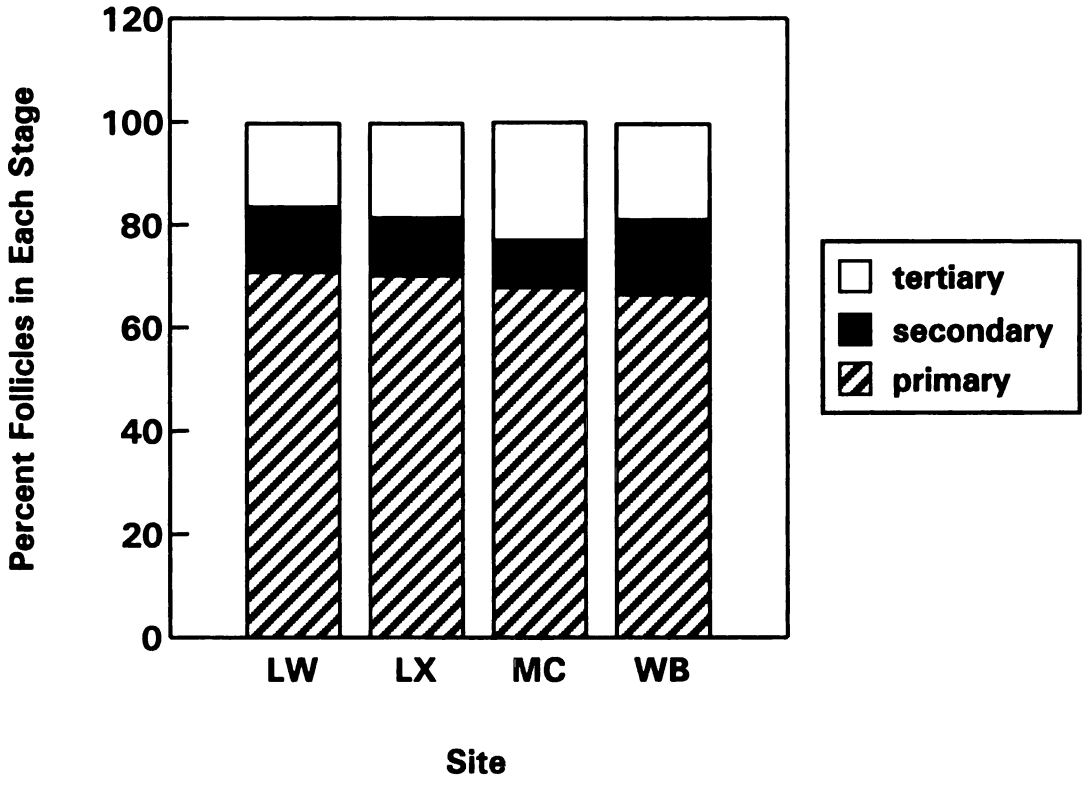

Figure 3-6. Percentages of ovarian follicles in each of three stages: primary, secondary, and tertiary. Bar segments represent the mean percentage of follicles in each stage.

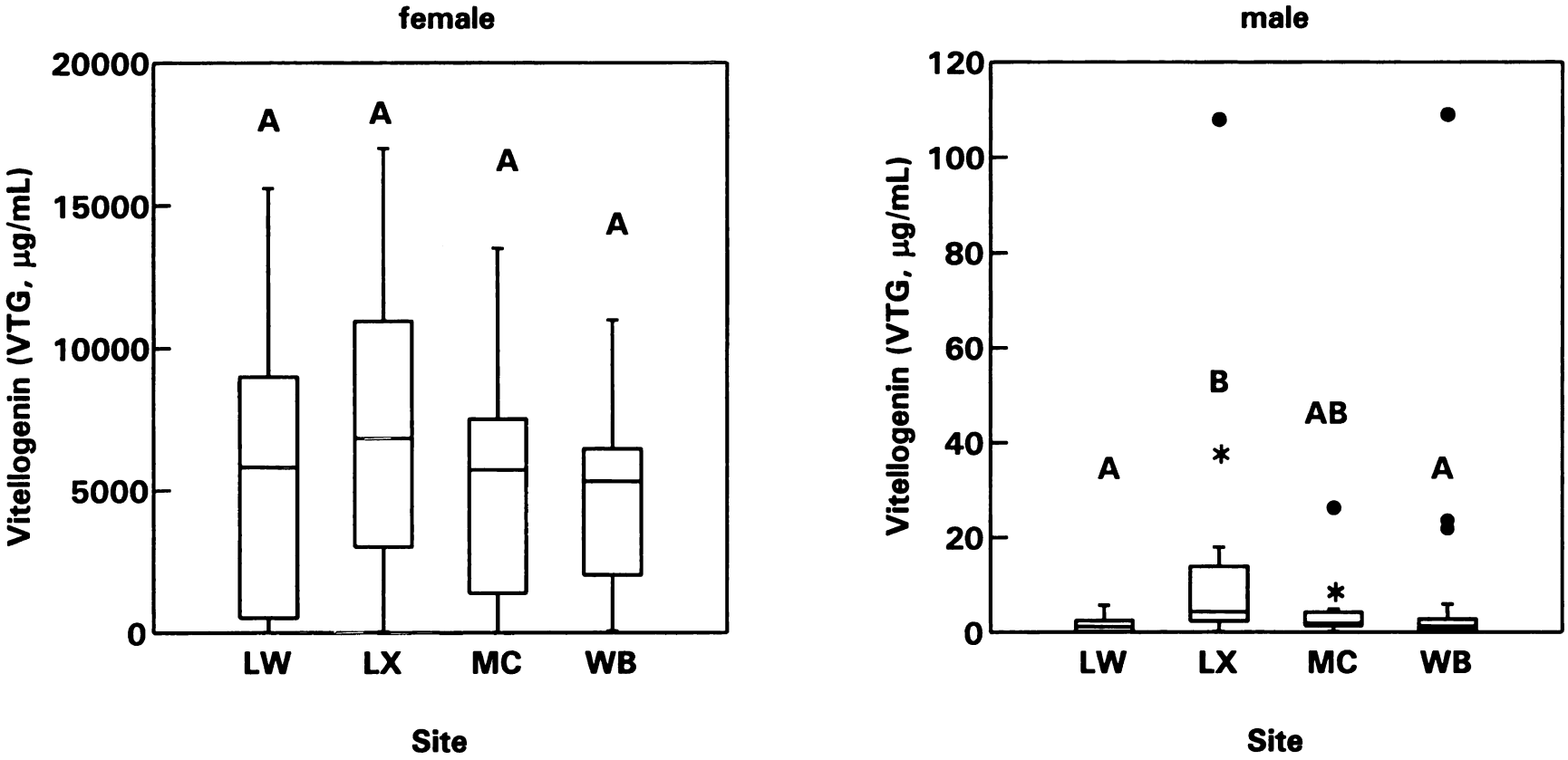

Figure 3-7. Plasma vitellogenin (VTG, µg/mL) concentrations in adult female and male carp. Letters above boxes represent Tukey-like groupings within each sex. The center horizontal lines mark the medians for each site, and upper and lower edges of the boxes (hinges) represent first and third quartiles. Whiskers show the range of observed values that fall within the inner fences. Asterisks mark "outside values" (those between the inner and outer fences), and circles mark "far outside values" (those beyond the outer fences). See text under **"Statistical analyses"** for discussion of terms.

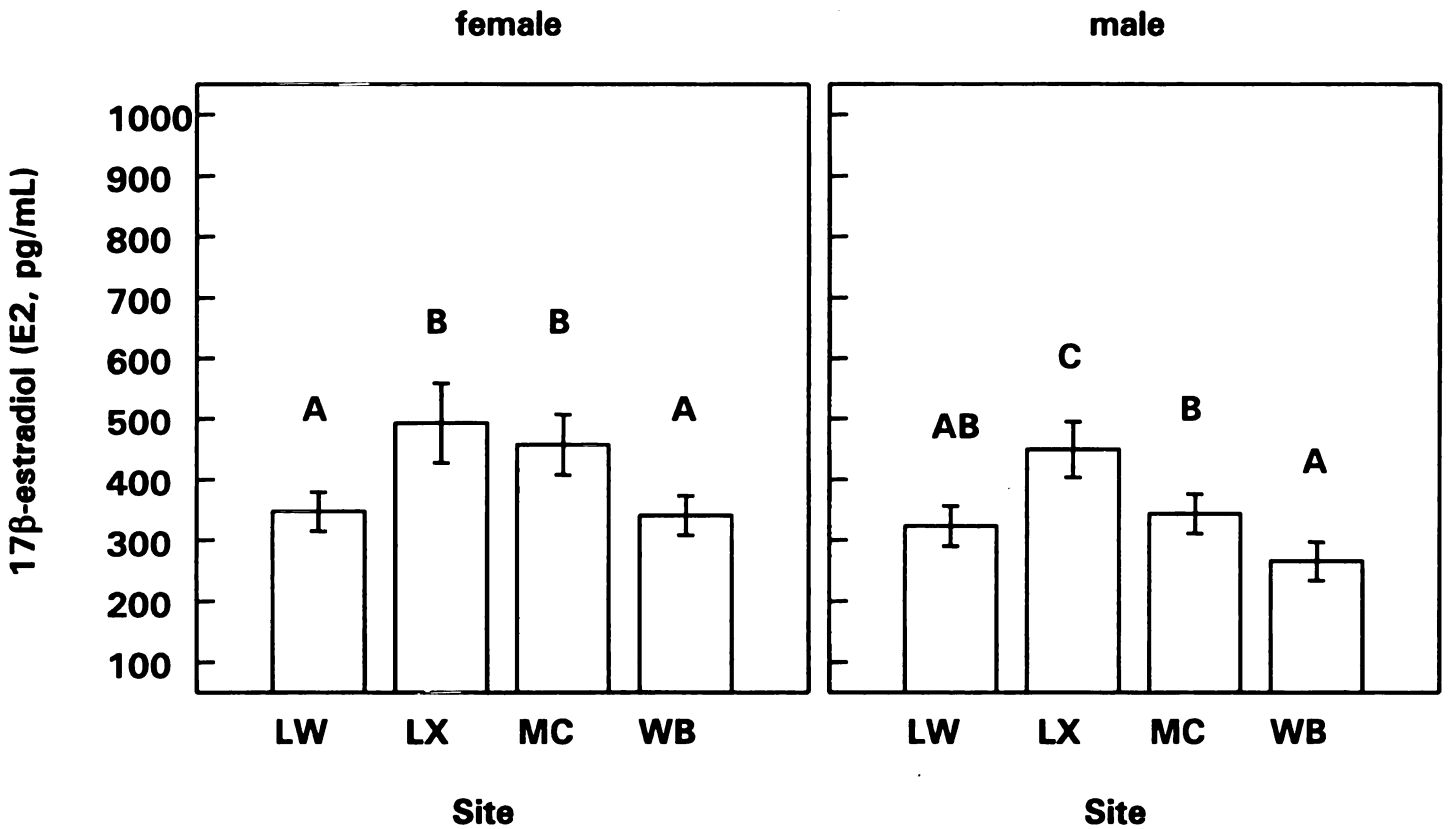

Figure 3-8. Plasma 17β-estradiol (E2, pg/mL) concentrations in adult female and male carp. Letters above the bars represent Tukey groupings within each sex. Error bars are 2 SEM. The sexes differed significantly in mean plasma E2 concentration within a site only at reference sites MC and WB.

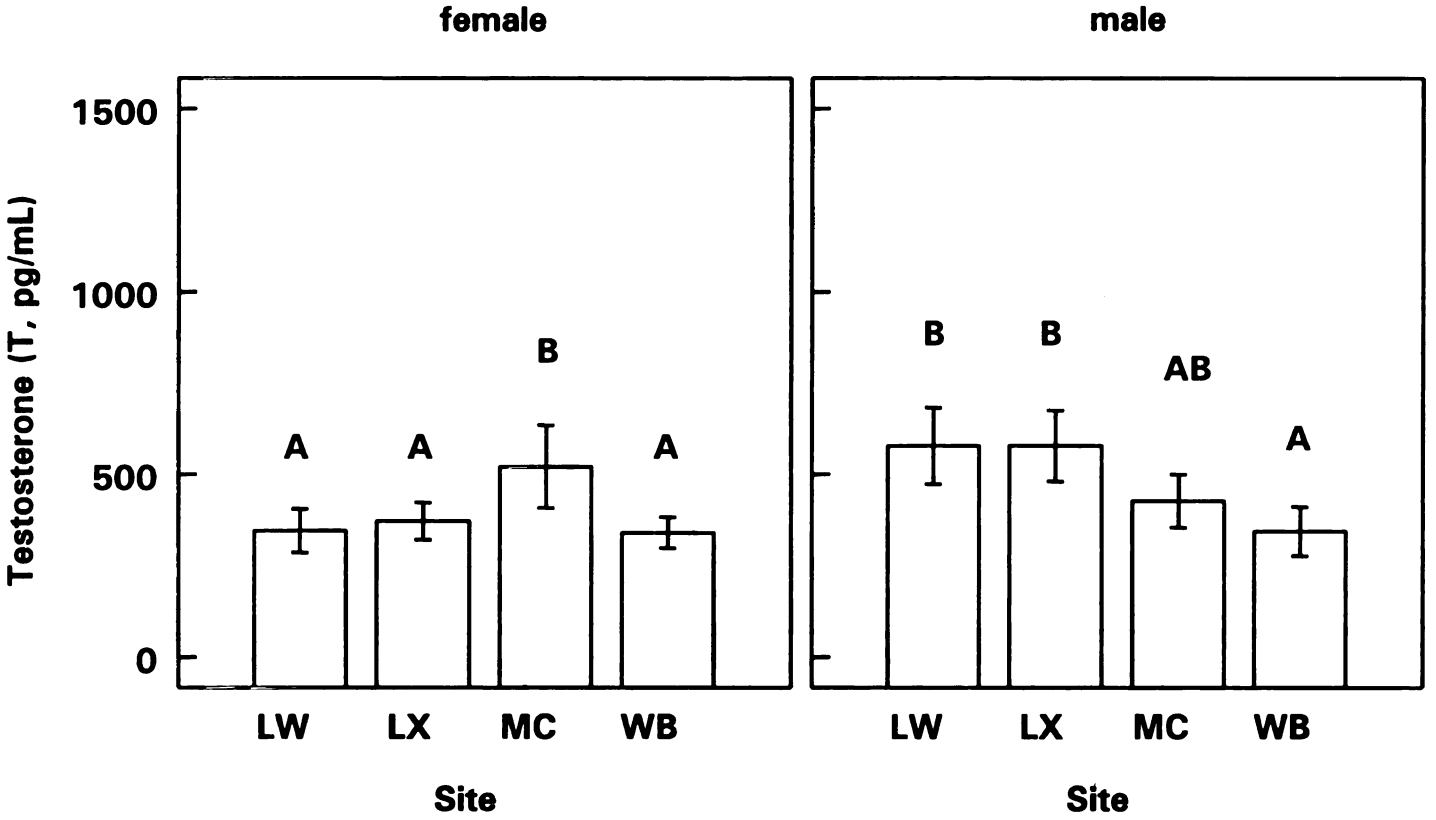

Figure 3-9. Plasma testosterone (T, pg/mL) concentrations in adult female and male carp. Letters above bars represent Tukey groupings within each sex. Bar heights represent site means, and error bars are 2 SEM. The sexes differ significantly within a site only at LW and LX.

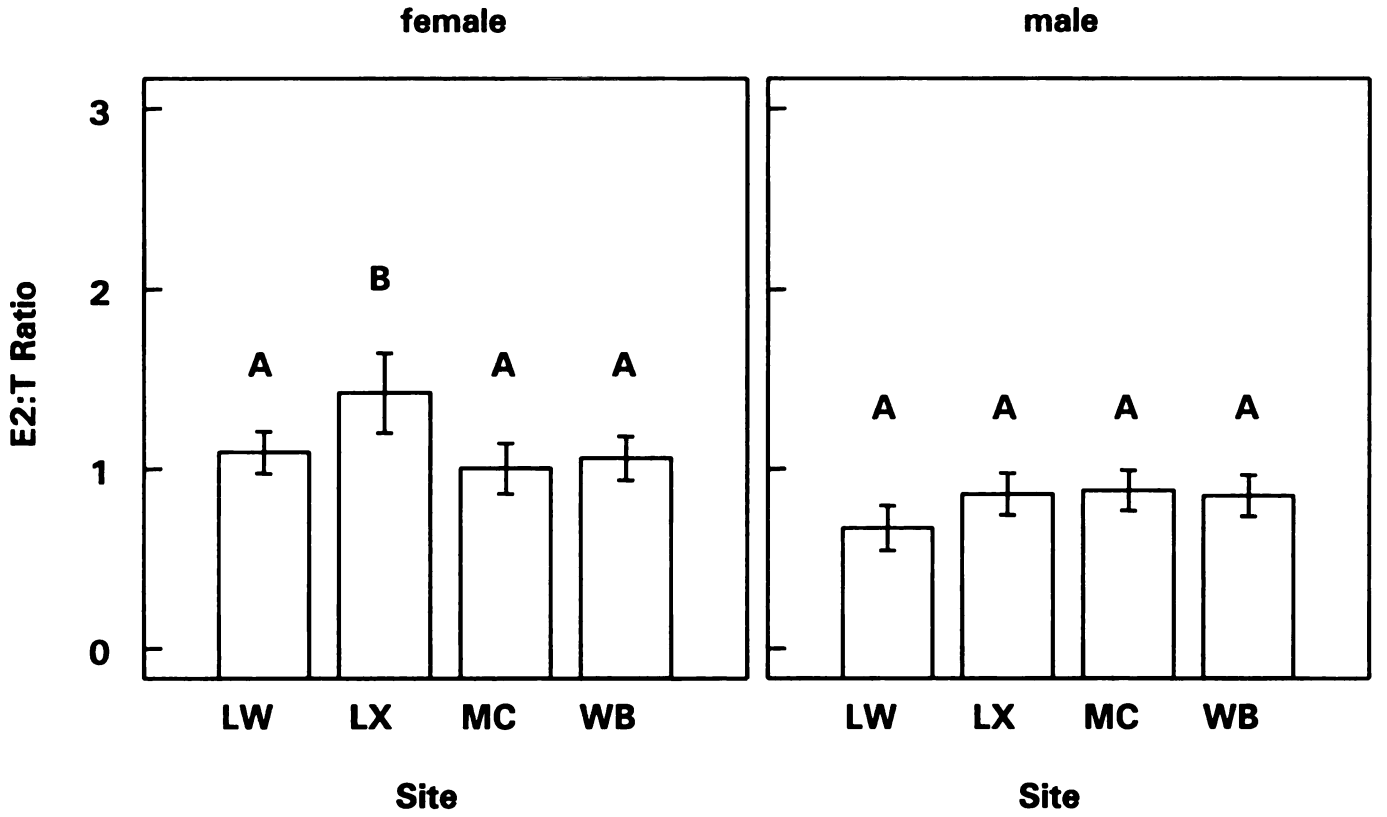

Figure 3-10. Ratio of plasma 17β-estradiol to plasma testosterone (E2:T) in adult female and male carp. Letters above bars represent Tukey groupings within each sex. The bar heights represent site means, and error bars are 2 SEM. The sexes differed significantly in site mean E2:T among fish caged at the same site only at LX and LW.

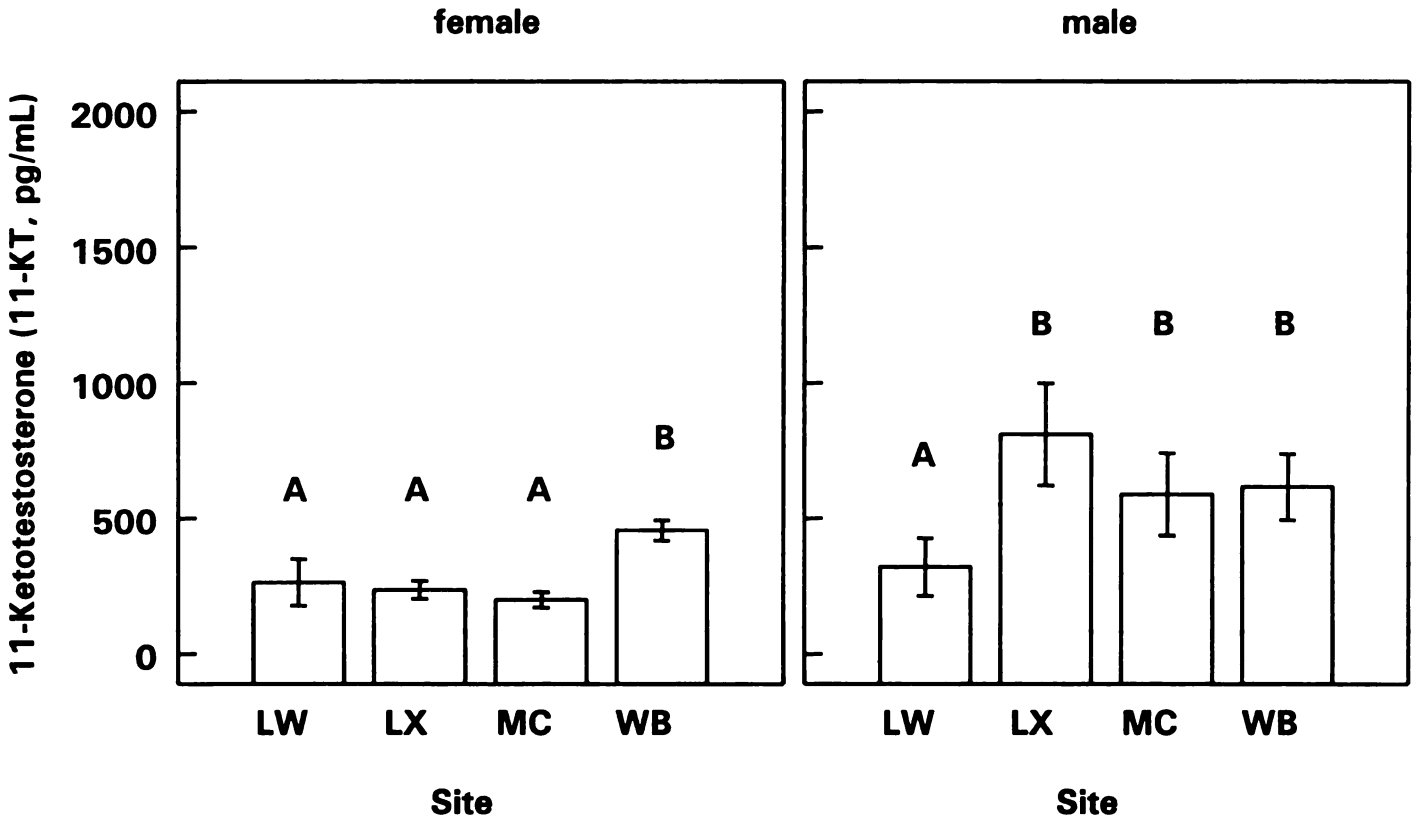

Figure 3-11. Plasma 11-ketotestosterone (11-KT, pg/mL) concentrations in adult female and male carp. Letters above bars represent Tukey groupings within each sex. Males and females caged at the same site differed significantly in mean plasma 11-KT concentration only at LX and MC.

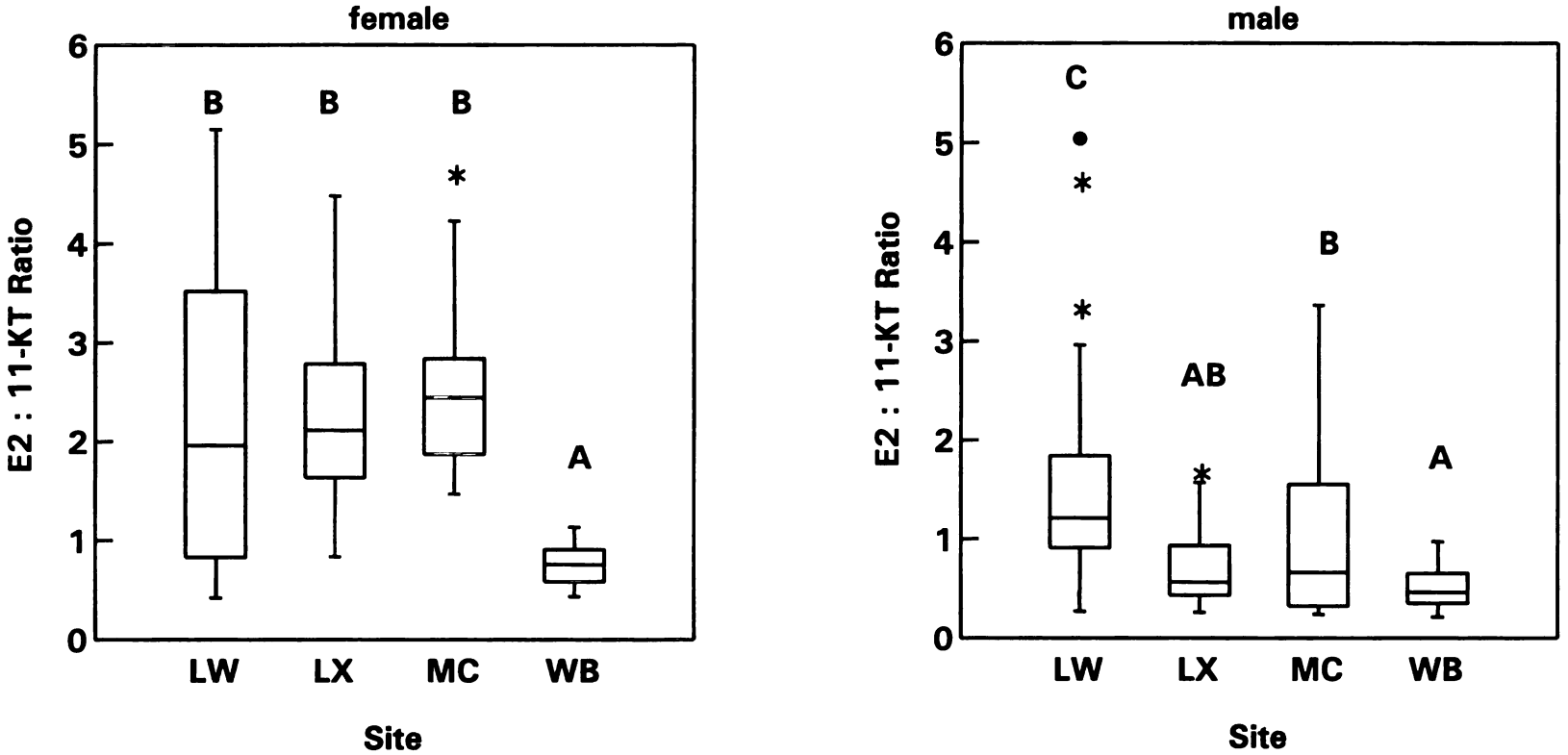

Figure 3-12. Ratio of plasma 17β-estradiol to plasma 11-ketotestosterone (E2:11-KT) in female and male carp. Letters above boxes represent Tukey-like groupings within each sex. The center horizontal lines mark the medians for each site, and upper and lower edges of the boxes (hinges) represent first and third quartiles. Whiskers show the range of observed values that fall within the inner fences. Asterisks mark "outside values" (those between the inner and outer fences), and circles mark "far outside values" (those beyond the outer fences). See text under **"Statistical analyses"** for discussion of terms. Fish of different sexes caged at the same site differed significantly in median E2:11-KT at all sites but LW.

APPENDIX A

HISTOLOGICAL EXAMINATION OF FISH GONAD AND HEPATOPANCREAS
Version 4.0
Updated July 15, 2000

Erin M. Snyder
Department of Zoology and Institute of Environmental Toxicology
Michigan State University

Dr. Scott Fitzgerald
Animal Health Diagnostic Laboratory and Department of Pathology
Michigan State University

I. Tissue fixation

Fish gonad and hepatopancreas samples are taken for histological examination. Fresh tissues are fixed in 10% neutral-buffered formalin (NB formalin) (see Appendix 2). Tissue samples should be no thicker than 1 cm, because this is the maximum penetration of fixatives. Samples thicker than 1 cm will decompose. Tissues that tend to have a similar superficial appearance after fixation should be stored in separate containers to avoid confusion when tissues are trimmed. Polypropylene centrifuge tubes (50 mL) work well for fixing tissues from carp and goldfish and for fixing entire fathead minnows or Japanese medaka. Tubes should have plug-type screw caps to prevent leakage. For larger tissues, wider pathology sample containers should be used.

II. Sample shipment

Federal Express (FedEx) considers formalin solutions of 10% or less nonhazardous and will ship them. All sample container lids should be checked for a good seal and then sealed in a secondary container such as a heat-sealed bag. Centrifuge tubes can be packed in their original foam racks and bags.

III. Tissue trimming and sample submission to the Histology Laboratory, Michigan State University

Tissue cassettes with lids can be obtained from the Histology Laboratory (see Appendix 3). If the tissue samples are small, as is frequently the case with goldfish gonad, obtain foam biopsy pads (small, square, blue sponges) to place in the cassettes. These will prevent the tissues from shifting in the cassettes if the orientation of the sample is important and will keep tissues from slipping out of the cassettes. Some tissues will shrink during fixation. Tissue samples should be trimmed with a very sharp blade to the thickness of a dime and placed in the tissue cassettes on top of the foam biopsy pads, with no more than three tissues per cassette. For goldfish, both hepatopancreas and gonad samples can be placed on the same slide. Tissue cassettes should be pre-labeled in pencil or solvent-resistant pen provided by the Histology Laboratory. Other pens, permanent markers, etc., cannot be used because the fixative will remove the marking, rendering the samples useless. Trimmed tissues in cassettes are submerged in NB formalin in a tightly closed, labeled container with a carcinogen warning label and submitted to the Histology Laboratory. It is recommended that a sample ID sheet accompany all submitted samples. At the Histology Laboratory, the tissues are embedded in paraffin, sectioned at 5 μm, and stained with haematoxylin and eosin. Turnover time for samples is generally about two weeks, but may take significantly more or less time.

IV. Histologic criteria

IV. A. Hepatopancreas

Slides of hepatopancreas tissue are examined for signs of necrosis, inflammation, neoplasia, foci of atypia, biliary stasis, and cellular degeneration. Vacuolar degeneration of hepatocellular cytoplasm (hepatocellular vacuolation) is a nonspecific lesion and indicates accumulation of glycogen and/or fat due to overfeeding, emaciation (use of fat stores), toxification due to various substances, and other causes. Hepatocellular vacuolation is graded on a scale as follows: 0 = no vacuolation, 1 + = mild vacuolation with small vacuoles spread throughout the cytoplasm, 2 + = moderate vacuolation with larger coalescing vacuoles appearing as large clear zones in many hepatocytes, 3 + = severe vacuolation where all or most of the cytoplasm has lost its normal pink coloration due to confluent, large, clear vacuoles.

IV. B. Ovary

Stages of ovarian follicle development are assessed as described by Miles-Richardson *et al.*, (1998). A typical area of the ovary is selected, and 50 follicles are counted within that area. Proportions of primary, secondary, tertiary, and atretic follicles per 50 follicles are recorded. Atretic follicles are unovulated follicles undergoing atresia, a process of degeneration.

Follicle Type	Description
Primary	abundant basophilic cytoplasm (deep blue color), large pale central nucleus, no yolk vesicles
Secondary	eosinophilic yolk vesicles appear in vacuolated cytoplasm, granulosa cells surround central oocyte
Tertiary	largest follicle type, numerous eosinophilic yolk globules fill cytoplasm
Atretic	degenerative follicle with collapsed, irregular contour; macrophages invading follicle

IV. C. Testis

Testis slides are scanned at 20X and 40X objectives, and the relative number and prominence of Sertoli cells in comparison to control fish are estimated using the following scale: 0 = no proliferation, 1 = mild proliferation (<one third), 2 = moderate proliferation (one third to two thirds) , 3 = severe proliferation (> two thirds). Degenerative changes (indicated by designation " +deg") could include germ cell syncytia, mineralization of spermatozoa, and variably sized or necrotic spermatozoa (Miles-Richardson *et al.*, 1999). Testes also are designated as spermatogenically active or inactive. Prior to dissection and sampling of testes for histology, male fish should be assessed for spermiation by stripping for milt, and sperm release should be recorded. These data can be used in conjunction with spermatogenic activity data to assess state of testicular development.

V. Statistical analyses and data presentation

Analyses of resulting data can be performed with the SAS System for Windows© Release 7.00 (SAS Institute, Cary, North Carolina, 1998) or

SYSTAT© Version 9 (SPSS Inc., Chicago, Illinois, 1998). For Sertoli cell proliferation and hepatopancreas vacuolation, the data are ranked and analysis of variance (ANOVA) is conducted on the ranks of the data or the data are subjected to a Kruskal-Wallis nonparametric ANOVA. A Tukey-like test or other suitable post-hoc comparison of medians also can be performed on these data. Follicle proportion data may be graphically represented as stack diagrams, which can be designed with SYSTAT©. Follicle count data also may be examined by use of multivariate profile analysis, which can be conducted with SAS©.

VI. References

Miles-Richardson, Stephanie R.; Kramer, Vincent J.; Fitzgerald, Scott D.; Render, James A.; Yamini, Behzad; Barbee, Steven J.; Giesy, John P. 1999. Effects of waterborne exposure of 17β-estradiol on secondary sex characteristics and gonads of fathead minnows (*Pimephales promelas*). *Aquatic Toxicology* 47: 129-145

Addendum 1

Abbreviations:

gal = gallon

g = gram L = liter

mL = milliliter

cm = centimeter

etc. = et cetera

Addendum 2

10% Neutral-buffered Formalin

Makes 1 gal or 3.8 L. Mix under a fume hood in a container that holds > 1 gal.

Caution: Formalin is a carcinogen. Wear gloves and other appropriate protective equipment and do not breathe vapors.

400 mL formalin*
16 g sodium phosphate monobasic ($NaH_2PO_4 \bullet H20$)
26 g sodium phosphate dibasic (Na_2HPO_4)

Bring up to one gallon with distilled water. Check the pH and adjust to about 7.

* Or formaldehyde solution, usually 37-50% aqueous solution of formaldehyde with up to 15% methanol

Alternatively, NB formalin can be purchased pre-made from Sigma.

Addendum 3

Histology Laboratory
A203 Clinical Center
Michigan State University

tel 517-353-9016
fax 517-432-1368

Contact person: Amy Porter

APPENDIX B

STANDARD OPERATING PROCEDURE

Measurement of Plasma Vitellogenin in Common Carp by Competitive Enzyme-linked Immunosorbent Assay (ELISA)

Adapted from a standard operating procedure authored by K. Nichols, Michigan State University

Erin M. Snyder
Michigan State University
Aquatic Toxicology Laboratory
Version 1.0
December 20, 1999

INTRODUCTION

This standard operating procedure (SOP) is a modification of a previous SOP developed by Nichols (1997) for measurement of vitellogenin (VTG) in goldfish and fathead minnow plasma. This protocol was developed for measurement of VTG in common carp plasma and includes some modifications for preventing the degradation of VTG during the analysis, including the use of lower assay temperatures. The two incubation periods at 37 °C in the original SOP were eliminated in favor of longer incubation periods at lower temperatures. Please refer to the original protocol for background information on the original development of the assay and polyclonal rabbit anti-goldfish VTG antiserum used in this SOP.

SAMPLE COLLECTION AND STORAGE

VTG is extremely sensitive to proteolysis, so care should be taken to minimize degradation of the protein by keeping samples cold and treating with a protease inhibitor. Blood plasma samples taken for analysis of VTG should be collected as quickly as possible from anesthetized or freshly killed fish before the blood begins to clot. Fish blood tends to clot rapidly, making collection of sufficient samples difficult. Collection of blood samples with heparinized hematocrit tubes or heparinized needles and syringes can alleviate this problem. If syringes are used to collect blood, they should be

chilled on ice prior to sample collection. Blood is placed immediately into a tube pretreated with aprotinin, a protease inhibitor, and gently mixed. Generally 1-2 TIU (trypsin inhibition units) per mL of blood collected is sufficient to protect common carp VTG from proteolysis if the samples are kept at 4 °C (N. Denslow, personal communication; Tyler et al., 1996). Blood sample tubes are placed on ice immediately and/or stored in a refrigerator for no more than 4 hr prior to centrifugation. Blood samples are centrifuged at 3000 × g for 10 min at 4 °C. The plasma is removed and frozen in working aliquots in Eppendorf vials at -80 °C until analysis. Samples are thawed and diluted in buffer immediately before analysis.

EQUIPMENT AND APPARATUS

- 96-well plate Spectra Reader plate-reading spectrophotometer with 492 nm and 650 nm filters (OEM Version; Cayman Chemical Autoreader, Cayman Chemical, Ann Arbor, MI)
- 200 µL multi-channel micropipetter
- incubator (25 °C)
- micropipetters (2 µL, 10 µL, 20 µL, 100 µL, 200 µL, 1000 µL, 5000 µL)
- Eppendorf repeating pipetter
- plate shaker
- automatic pipetter

MATERIALS

Chemicals

- 1,2-phenylene diamine or *o*-phenylene diamine (OPD, Sigma P-3804)
- 30% hydrogen peroxide (Baker 2186-01)
- ammonium acetate (Baker 0596-01)
- citric acid (Columbus Chemical Industries, Inc. 11993-010)
- gentamicin solution (Boehringer Manheim 1059467)
- normal goat serum (Sigma G-9023)
- sodium bicarbonate (Baker 3506-01)
- sodium chloride (NaCl, Baker 3624-05)
- sodium hydroxide (NaOH)
- hydrochloric acid (HCl)
- sulfuric acid (H_2SO_4)
- Tris (Gibco 15504-012)
- ultrapure water (Barnstead or Milli-Q water)
- Tween-20 (Sigma P-7949)

- donkey anti-rabbit IgG conjugated to horseradish peroxidase (Amersham International NA934)
- rabbit anti-goldfish vitellogenin polyclonal antiserum

Supplies

- 12 X 75 mm disposable glass test tubes
- 96-well flat bottom high binding ELISA plates (Costar 9018, Bio-Rad 224-0096)
- Eppendorf repeating pipetter CombitipsPlus, 2.5 mL (Brinkmann 22 26630-6) and 5.0 mL capacity (Brinkmann 22 26640-3)
- paper towels
- pipette boats
- plastic wrap
- aluminum foil
- laboratory timer
- automatic pipetter tips (Costar 4101)
- micropipetter tips (1-10 µL, 10-200 µL, 200-1000 µL)
- small beakers
- media bottles

ELISA BUFFERS AND REAGENTS

Notes on buffer preparation:

- All buffers should be prepared prior to beginning the VTG ELISA with the exception of the AACA solution for color development. SBB and TBST may be stored at 4 °C for up to 3 months, but the pH must be checked and adjusted on each day that the buffers are used. Also, the pH meter should be calibrated to pH 10, then pH 7 prior to checking buffer pH. TGST-SG should be discarded after 24 hr. Ammonium acetate and citric acid solutions can be made in excess and stored in separate bottles for an extended period of time at room temperature or at 4 °C.
- When preparing buffers, one should dissolve solids in a volume slightly less than the total volume desired, adjust the pH, then fill to volume.
- The pH of Tris buffers is temperature-dependent, so check the pH of the solution at the temperature at which it is to be used.
- Wear gloves throughout buffer preparation both for personal protection and to prevent proteins and oils on hands from interfering in the assay.
- Use caution when handling corrosive liquids like citric acid solution, NaOH, and HCl. OPD is toxic and should not be handled without gloves.

Sodium bicarbonate buffer (SBB)
(50 mM sodium bicarbonate, 5 mg/L gentamicin)

sodium bicarbonate (NaHCO3)	4.20 g
gentamicin solution	100 µL (=5 mg)
ultrapure water	fill to 1000 mL

Adjust to pH 9.6 with 1 M NaOH or HCl.
Store at 4 °C. Check and adjust pH before use.

10X Tris buffered saline (10X TBS)
(100 mM Tris, 1.5 M NaCl)

Tris	12.11 g
NaCl	87.66 g
ultrapure water	fill to 1000 mL

Adjust pH to 7.5 with 1 M NaOH or HCl.
Store at 4 °C or at room temperature.
Check and adjust pH before use.

Tris buffer with Tween-20 (TBST)
(10 mM Tris, 0.15 M NaCl, 0.1% Tween-20, 5 mg/L gentamicin)

10X TBS	100 mL
Tween-20	1.0 mL
gentamicin solution	100 µL (=5 mg)
ultrapure water	fill to 1000 mL

Adjust pH to 7.5 with 1 M NaOH or HCl.
Store at 4 °C. Check and adjust pH before use.

Blocking buffer / Sample buffer (TBST-SG)
(TBST with 2% goat serum)

normal goat serum	2.0 mL
TBST	98.0 mL

Mix just prior to use and discard after 24 hr.
300 mL is enough for 2 plates plus excess for sample dilution.

Ammonium acetate - citric acid solution (AACA)
used to make OPD solution (see below)

(1) ammonium acetate solution (50 mM)

ammonium acetate	0.385 g
ultrapure water	fill to 100 mL

(2) citric acid solution (50 mM)

citric acid	0.525 g
ultrapure water	fill to 50 mL

Measure the amount of solution 1 needed for the number of plates to be run (see below), then adjust solution 1 to pH 5.0 with solution 2.

OPD solution	1 plate	2 plates	3 plates
o-phenylene diamine (OPD)	10 mg	15 mg	25 mg
30% hydrogen peroxide	10 µL	15 µL	25 µL
AACA solution	20 mL	30 mL	50 mL

Note: OPD is **toxic**.

HAZARDS AND PRECAUTIONS

Strong acid and strong base solutions (citric, hydrochloric, and sulfuric acids and sodium hydroxide) are caustic; wear appropriate gloves, protective clothing, and safety glasses or goggles. OPD is toxic and should not be handled without gloves.

VTG in the purified standard solutions and in the samples is extremely sensitive to proteolysis at temperatures above 4 °C and when subjected to repeated freezing and thawing. Immunochemicals and antisera also are sensitive to degradation particularly due to freezing and thawing. All antisera, standard protein solutions, normal goat serum, and samples should be stored at -80 °C in working aliquots and should be thawed immediately prior to use. Also, these should not be exposed to room temperature for long periods of time before the assay is run. Diluted immunochemicals, antisera, and samples can be stored for one day at 4 °C. Also, proteins such as VTG, antisera, and immunochemicals tend to bind to some plastics. Accordingly, on the day that they are diluted for use, these materials should be diluted and stored for no more than 1 day in glass containers. Plastic pipette tips should not be left inside the solutions for more than the brief time it takes to dispense them.

PROCEDURE

This procedure describes steps for running an assay on one 96-well plate. Generally, 3 to 4 plates can be run at one time if each plate is started about 1.5 hr after the last.

A. Sample and standard curve layout

Standard curves should be run in duplicate on each plate. Samples also should be run in duplicate or triplicate. Record the layout of standards and samples on a plate layout form before moving on to the next step. If plates are always run in the same format, an Excel macro can be used to analyze the results. If multiple plates will be run for samples that are to be compared to one another, the same sample should be run on each plate for calculation of inter-plate variation. Wells should be set aside for 10 standards, maximum binding, nonspecific binding (NSB), and inter-plate variation samples.

B. Plate coating

1. Remove SBB from the refrigerator. Check the pH of SBB and adjust to pH 9.6.
2. Aliquots of purified carp VTG are stored at -80 °C. Remove one aliquot (10 µL/aliquot, 3.037 mg VTG/mL) of purified carp VTG (VTG standard) from the freezer and thaw at room temperature.
3. Pipet 1.47 µL of standard VTG solution (in this case, the standard labelled "I") into 15 mL chilled SBB in a small glass beaker (a glass scintillation vial works well) and mix well by gently vortexing or aspirating with repeating pipetter.
4. Pipet 150 µL of diluted VTG standard into each well of a 96-well plate to achieve a coating of 45 ng VTG/well. Cover the plate tightly with plastic wrap and leave at 4 °C for 24 hr. (Note: Keep coating and incubation times as consistent as possible. Wait 1-1.5 hr before coating the next plate so that later steps can be completed in a consistent time frame by one worker.

C. Standard curve dilutions

If a 24 hr plate coating time is used, standard curves are prepared the next day.

1. Calibrate the pH meter to pH 10, then pH 7. Check the pH of TBST and adjust to pH 7.5. Prepare TBST-SG.

2. Number ten 10 X 75 mm glass test tubes 1-10 for standards. Pipet 2.00 mL of TBST-SG into tube 1. Pipet 1.00 mL of TBST-SG into the remaining tubes.

3. Thaw another aliquot of standard VTG solution. Pipet 1.8 µL of standard VTG solution into tube 1 for the greatest standard (2733 ng/mL). Vortex tube 1 gently, then transfer 1.00 mL from tube 1 to tube 2. Repeat this process for the remaining tubes to make serial dilutions (vortex tube 2, transfer 1.00 mL to tube 3, etc.)

Tube #	µL/well	ng/mL
1	50	2733
2	50	1367
3	50	683.3
4	50	341.6
5	50	170.8
6	50	85.41
7	50	42.70
8	50	21.35
9	50	10.68
10	50	5.338

4. Place standards in the refrigerator until needed. Standards can be used for up to 24 hr before new ones should be prepared if they are kept at 4 °C.

D. Sample dilutions

Samples are diluted at least 1:50 to avoid serum effects.

This gives the ELISA a method detection limit (MDL) of 0.267 µg VTG/mL.

Typically, plasma samples taken from male fish are diluted 1:50 to start, and plasma samples taken from female fish are diluted 1:1000 to 1:5000 to start. After the first samples are run, better judgements can be made to determine what dilution will place the majority of samples for each sex in the range of the standard curve.

1. Plasma samples are removed from storage at -80 °C and thawed at room temperature or in a refrigerator.
2. Dilutions are made in 12 X 75 mm disposable glass test tubes. Add the appropriate volume of TBST-SG to each tube, then add plasma and vortex gently. Diluted plasma samples can be kept in a refrigerator for a brief period of time until needed.

3. Dilutions can be made as follows:

dilution	volume plasma	volume TBST-SG
1:50	4.00 µL	196.0 µL
1:1000	1.00 µL	999.0 µL
1:5000	0.50 µL	2500 µL

E. Plate washing

1. Discard the contents of all wells in sink and pat plate on paper towels to remove standard VTG solution.
2. Wash wells 4 times with 200 µL TBST per well using a multi-channel pipetter. Pat the plate on paper towels after each wash.

F. Blocking

The remainder of the well surface not covered by purified standard VTG is blocked by saturating with proteins in normal goat serum.

1. Add 200 µL TBST-SG (blocking buffer) to each well with a repeating pipetter.
2. Cover the plate with plastic wrap and incubate at 25 °C for 3 hr. Use an incubator to avoid variability in blocking due to fluctuations of room temperature.
3. Discard the TBST-SG from the wells into sink and pat the plate on paper towels to remove the buffer remaining in the wells. Do not wash.

G. Addition of samples, standards, and primary antiserum

1. Dilute the primary antiserum (rabbit anti-goldfish VTG polyclonal antiserum) in TBST-SG in a glass beaker. For the current study, the antiserum identified as vg927rb6 was used at a dilution of 1:45000. Antiserum was originally diluted 1:5 and frozen in 10 µL aliquots, so 2.50 µL of the diluted antiserum was added to 22.5 mL TBST-SG to achieve a dilution of 1:45000. Place diluted antiserum in the refrigerator until needed. It can be used for up to 24 hr if kept at 4 °C.
2. For the greatest consistency, use the same pipet tip to pipet buffer into maximum binding wells and to pipet standards. Pipet 50 µL TBST-SG into each of the maximum binding wells, then use the same tip to pipet 50 µL of standard 10 (least concentration) into each of the duplicate wells, etc., to standard 1 (greatest concentration).

3. Add 150 µL TBST-SG to each of the NSB wells.
4. Add 50 µL diluted sample to each of the duplicate wells for samples.
5. Add 100 µL diluted primary antiserum to all wells except NSB wells.

DO NOT ADD PRIMARY ANTISERUM TO NSB WELLS!!!

Note that the dilution of antiserum used here was designed to achieve 50% maximum optical density (OD) at the middle of the standard curve. Primary and secondary antiserum dilutions should be tested with this in mind when different batches of antisera are used.

6. Shake the plate briefly to mix the contents of the wells. Cover the plate tightly with plastic wrap (or alternatively, plate sealing film or a sealing lid) and incubate 12 hr at 25 °C in the incubator.

H. Plate washing

Proceed as in step E. This step removes sample and unbound primary antiserum from the wells.

I. Addition of secondary antiserum

1. Dilute secondary antiserum (donkey anti-rabbit IgG conjugated to horseradish peroxidase) at 1:2000 in TBST-SG. Add 10 µL secondary antiserum to 20 mL of TBST-SG in a glass beaker. Mix thoroughly. The undiluted secondary antiserum can be frozen in working aliquots at -80 °C for use over long periods of time. However, it also can be kept for long periods of time (3 months) at 4 °C for assays that will be completed in that length of time. Check the expiration date on the vial when it arrives to judge whether it should be frozen. After dilution, secondary antiserum can be used for up to 24 hr if kept at 4 °C.
2. Add 150 µL diluted secondary antiserum to each well with a repeating pipetter.
3. Cover plates tightly in plastic wrap and incubate for 2 hr at 25 °C.

J. Plate washing

Proceed as in steps E and H. Wash 5 times instead of 4 times to reduce nonspecific binding.

K. Color development

1. Mix the AACA solution with the appropriate amount of OPD and then add the hydrogen peroxide just before use. Note: Light exposure will cause the OPD solution coloring reaction to occur regardless of the amount of VTG present, particularly after hydrogen peroxide is added. Protect the solution from light by covering with aluminum foil, placing in a dark drawer, or turning lights down, as appropriate.
2. Turn down one bank of lights if possible to prevent yellow color from developing due to light exposure. Quickly add 150 µL OPD solution into each well with a repeating pipetter.
3. Cover plate with plastic wrap, then with aluminum foil to block out light. Shake the plate on the plate shaker for 30 min to allow color to develop in the presence of horseradish peroxidase bound to the secondary antibody.

L. Stop coloring

1. Add 50 µL of 5 M sulfuric acid to each well with a repeating pipetter.
2. Shake the plate on a plate shaker for 10 min in the dark.

M. Optical density (OD) measurement on a plate-reading spectrophotometer

1. While the plate is being shaken, turn on the computer and plate-reading spectrophotometer. The software for the spectrophotometer is Cayman EIA software Version 2.0. Verify that the spectrophotometer contains filters for reading 492 nm and 650 nm.
2. Go to "Read a Plate" in the main menu and check settings.
   <F4> Reader Specific Options: Choose dual wavelength and enter 492 nm for the measured wavelength and 650 for the reference. Turn blanking off.
   <F1> Plate ID: name the plate so that the software does not overwrite another file.
3. Gently wipe any smudges from the bottom of the plate with a soft cloth. Do not use Kimwipes because they can score the plastic. Make sure the plate orientation is correct and scan the plate.
4. Print the data by entering **<F8> Print file to printer** and save to a disk or to the hard drive under C:\Cayman.

N. Data analysis

1. Microsoft Excel can be used to open the data file. If plate layouts are always the same, a macro can be used to analyze the data. Average the duplicate OD readings for each sample, standard, maximum binding wells, and NSB wells and use the average value for further calculations. For each standard and sample, calculate the percent bound and logit OD as follows:

   % bound = (sample OD - NSB OD) / (maximum binding OD - NSB OD)

   logit OD = log [(% bound) / (1-% bound)]

   Calculate the percent coefficient of variation (%CV) for all duplicate OD measurements of samples as:

   %CV = (standard deviation / mean) × 100

   Any sample with %CV > 10 is re-assayed.

2. In the spreadsheet, plot the standard curve with log VTG concentration (ng/mL) on the x-axis and logit OD on the y-axis. Perform a linear regression and use the resulting equation to determine sample concentrations of VTG. The r^2 for the regression equation should be 0.95 or greater. The logit transformation usually linearizes the standard curve but increases the error at the tails, so only samples that fall between 15% and 85% binding are considered to be reliable measurements. Samples that fall outside that range are re-assayed at a different dilution. If a sample is assayed at the minimum dilution (1:50) and produces >85% binding, the VTG concentration is less than the method detection limit (MDL).

   The logit transformation is not necessary if the untransformed data routinely produce linear standard curves.

   The regression equation on the log/logit transformation of the standard curve is :

   logit OD = m * log [VTG in ng/mL] + b

   where m= slope of the standard curve regression line and b= y-intercept

Sample VTG concentrations are determined by:

[VTG in ng/mL] = 10^((logit OD - b)/m)

Correct this concentration for dilution by multiplying by the dilution factor. For example, if a sample is diluted 1:50, multiply the concentration of VTG in ng/mL by dilution factor 50 to determine the concentration in the original sample.

3. Inter-plate %CV is calculated on the mean of two duplicate measurements of the same sample on each plate as described by Grotjan and Keel (1996). An inter-plate %CV < 15% is an appropriate goal for this type of approach.

RECOMMENDATIONS

Because the pH of Tris buffers changes with temperature, future alterations in this protocol might include a switch to a phosphate buffer instead of Tris buffer.

The concentration of aprotinin that is needed to prevent degradation of VTG in plasma samples might be more thoroughly investigated. Other laboratories have used concentrations as great as 20 TIU/mL blood. In addition, it would be interesting to determine whether aprotinin added to ELISA buffers changes the results significantly.

Because aprotinin is so sensitive to degradation due to freezing and thawing, some other laboratories add glycerol to VTG standard solutions prior to freezing them. Glycerol protects the VTG from actually freezing at the low temperatures required to prevent microbial degradation of the standards. This method might be tested for use in our laboratory.

Commercial VTG kits will become available in the near future. Use of commercially available reagents might simplify the process and drastically reduce the amount of work involved in optimizing an ELISA for a new batch of polyclonal antiserum.

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ADDENDUM

N. Denslow provided much of the following information.

Aprotinin solution

Lyophilized aprotinin (Sigma A-1153) is dissolved in a solution of 0.9 % NaCl and 0.9% benzyl alcohol. Aprotinin solution is stored in a tightly sealed glass container at 4 °C for no longer than 1 month. This solution should be added to blood sample tubes before sampling. Use a concentrated solution so that a only a small volume must be added to the blood sample; 100 TIU/mL is suggested. Add 10 µL aprotinin solution per mL of blood to be sampled.

Heparin solution

A solution of 10 mg/mL ammonium heparin (Sigma H-6279) in 0.9% NaCl can be used to heparinize syringes. Fill and rinse void volume. Store at 4 °C.

APPENDIX C

PROTOCOL FOR ELECTROPHORESIS AND WESTERN BLOTTING OF VITELLOGENIN

Version 2.1
March 30, 2000

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INTRODUCTION

Vitellogenin is separated from other plasma proteins by gradient gel electrophoresis. Then the proteins are transferred to a nitrocellulose membrane. Polyclonal rabbit anti-goldfish vitellogenin antiserum developed in this laboratory is used to probe the membrane for vitellogenin by Western blotting (immunoblotting for proteins). The membrane is exposed sequentially to primary antiserum (polyclonal rabbit anti-goldfish vitellogenin), a secondary antibody (biotinylated donkey anti-rabbit IgG, whole antibody), and a streptavidin-peroxidase antibody. The primary antiserum binds to vitellogenin, the secondary antibody binds to the primary, and the streptavidin-peroxidase antibody binds to the biotin conjugate on the secondary antibody and to the biotin on biotinylated molecular weight markers to form a streptavidin-biotin complex. ECL (enhanced chemilumiscence) western blotting reagents are used to visualize the antibody complexes by detecting luminescence. The peroxidase catalyzes the oxidation, and consequent exicitation, of luminol in the reagents. When the luminol decays to ground state, it emits light which can be detected on autoradiography film or with the Bio-Rad ChemiDoc imaging system. Vitellogenin bands are identified by molecular weight (comparison to molecular weight markers) and reaction with the primary antiserum.

PROTEIN SEPARATION BY GEL ELECTROPHORESIS (SDS-PAGE)

Equipment and materials

- constant-current power supply (Bio-Rad Model 200/2.0)
- dry-block heating unit (Thermolyne Dri-Bath)
  or boiling water bath and Eppendorf tube rack
- electrophoresis apparatus (Mini-Protean II, Bio-Rad 165-2940)
- gel loading pipet tips
- micropipetters
- razor blades
- Eppendorf vials
- Tris-glycine (Tris-HCl) pre-cast gels (Bio-Rad Ready Gel, Bio-Rad 161-0902 or 161-1104; 4-15% Tris-HCl)
- Ready Gels Application Guide (Bio-Rad 161-0993)
- fume hood
- pH meter and calibration buffers
- stir plate and stir bars

Chemicals

- bromophenol blue (Sigma B-1026 or Bio-Rad M-3978)
- glycerol (SigmaUltra, G-6279)
- Milli-Q water
- biotinylated protein molecular weight markers (Bio-Rad 161-0319, broad range)
- sodium dodecyl sulfate (SDS, electrophoretic grade, Boehringer Manheim 1667262)
- Tris (Gibco 15504-012)
- hydrochloric acid (HCl, for pH adjustment)
- 2-mercaptoethanol (β-mercaptoethanol or BME; Sigma M-7154)
- glycine (Sigma G-7126)

Procedure

Sample Preparation

Dilute samples and molecular weight standards (MW standards) in Milli-Q water and **2X treatment buffer.** Samples should be diluted to achieve an appropriate protein load (1-10 μg total protein per well). MW standards are diluted according to the instructions that are provided with the product. Broad range markers are used here, but the high range markers also would be suitable. After samples and MW standards are diluted, heat them to 95

°C or place in boiling water bath for approximately 5 min. Samples and MW markers can be heated in plastic Eppendorf vials. Chill samples and MW markers to 4 °C and keep them chilled (on ice if possible) while they are pipetted into the gel wells.

TIPS:

For samples for which there is no good estimate of protein concentration, a protein assay, such as the Bradford assay, should be used to determine the dilution that will result in loading of 1 to10 μg total protein per well.

Use a well loading volume sufficiently large to allow accurate measurement of the volume but sufficiently small that it is easy to load the wells without spilling sample into the tank buffer or into adjacent wells. For 30 μL capacity wells, a volume of 10 μL works well.

Molecular weight standards- Use of biotinylated MW standards in this system will cause the MW standards to appear on the resulting film or image along with the proteins disclosed by the antibodies. This aids in determining the correct orientation of the image and in positive identification of the protein of interest and/or determination of the molecular weight of the proteins detected. In an automatic imaging system, software is available that can calculate the molecular weights of different bands. MW standards can be divided into aliquots and frozen, then thawed and diluted for later use.

Sample Loading and Electrophoresis

If using pre-cast gels, follow instructions in the Ready Gels Application Guide for gel preparation and placement into the electrophoresis tank with **tank buffer**. Use gel loading pipette tips to flush the wells with tank buffer to equilibrate them prior to loading samples. Make sure there are no air bubbles in the wells. Run the gels at 40-50 V for several hr until the bromophenol blue dye front begins to diffuse off the end of the gel into the tank buffer.

TIPS:

Samples are loaded with gel loading tips by placing the tip between the two plates and into the well. Release a small amount of sample into the well to make sure it falls to the bottom before adding the

remainder, to make sure the tip is actually in the well. Be careful not to add air bubbles to the wells.

The dye is low molecular weight and will reach the end of the gel before the proteins of interest.

If running two gels simultaneously, label the gels.

Both sides of the gel clamp must be screwed down, regardless of whether only one gel is run, to maintain a tight seal to avoid leakage of buffer between the upper and lower chambers.

Running the gel more slowly (at lower voltages) reduces "smiling," or curving of the bands.

To run two gels and two protein transfers, make about 4 L of tank buffer, two of which will be used to make transfer buffer.

PROTEIN TRANSFER TO NITROCELLULOSE MEMBRANE

Equipment and materials

- electrophoretic transfer cell apparatus:
  Mini Trans-Blot®, Mini-Protean II (BioRad 1703935); or
  Trans-Blot® SD Semi-Dry Electrophoretic Transfer Cell (Bio-Rad 170 3940)
- Bio-Ice cooling unit (Bio-Rad 170-3934) (if using Mini Trans-Blot system)
- constant current power supply (Bio-Rad Model 200/2.0, Bio-Rad 165-4761)
- nitrocellulose membrane (Hybond™ ECL™, Amersham Life Science RPN2020D)
- fiber pads (Bio-Rad 170-3933)
- filter paper (Bio-Rad 1703932)
- Hyperfilm™ autoradiography film (Amersham Life Science RPN2114H)
- ice
- micropipetters
- plastic or glass trays (or pipette tip tray lids)
- laboratory shaker
- SDS-PAGE gel containing vitellogenin
- refrigerator
- pH meter and calibration buffers
- stir plate and stir bars

Chemicals

- Tris (Gibco 15504-012)
- glycine (Sigma G-7126)
- sodium dodecyl sulfate (SDS, electrophoretic grade, Boehringer Manheim 1667262)
- hydrochloric acid (HCl, for pH adjustment)
- methanol (J.T. Baker 9093-03)
- Milli-Q water
- sodium chloride (NaCl, Baker 3624-05)
- Tween-20 (Sigma P-7949)
- nonfat dry milk
- Ponceau S (Sigma P-3504)
- acetic acid (EM Science, Gibbstown, New Jersey)

Procedure

The gel is removed from the gel clamp assembly and equilibrated in **transfer buffer**, keeping it in the same orientation as it was when the samples were loaded. A nitrocellulose membrane (NC) is cut to fit the precut filter papers. Hybond™ ECL™ Nitrocellulose Membrane is recommended because it is optimized for ECL reagents. Wet the NC in transfer buffer and place it on a shaker to soak it for at least 10 min (but not more than 30 min). Slide the membrane under the gel to make sure the orientation of the membrane will be the same as the orientation of the gel when the samples were loaded. Sandwich the NC and gel between two pieces of filter paper and then inside one or two fiber pads (provided with the Mini-Protean II system). Load the "sandwich" into the cassette so that the gel is toward the anode (black) side and the NC is toward the cathode (clear) side of the cassette. Load the cassettes so that the black side is toward the anode (black) in the tank. Set the power supply for 70 V and run for 4 hr. At this setting, the ice in the Biolce cooling unit must be changed periodically to prevent the buffer from overheating.

After the protein is transferred, proteins on the membrane can be stained with **Ponceau S** (reversible total protein stain), usually for 10 to 30 min, to determine whether proteins have transferred and to visualize protein bands that will not be detected by Western blotting. If non-biotinylated molecular weight markers are used, this stain will reveal the position of the bands of interest relative to the molecular weight markers. After staining with Ponceau S, the NC is destained with Milli-Q water until the background is white but not so long that the proteins bands destain. After photographing and/or marking molecular weight markers, destain the NC completely with water.

The NC is rinsed quickly 3 times with **Tris-buffered saline and Tween-20** (TBST) and placed in TBST. At this point, the procedure can be stopped until the next day. If desired, the NC may be blocked overnight by placing it in **blocking buffer** and storing at 4 °C. Blocking buffer should be mixed immediately before use.

TIPS:
If desired, trim one corner of each gel and its corresponding NC to make sure the orientation is not confused.

If the gels tend to stick to the filter paper, place a piece of nitrocellulose membrane between the filter paper and gel on the anode side as well as the cathode side. The proteins should still migrate onto the membrane nearest the cathode.

Blocking overnight may decrease nonspecific binding. If overnight blocking is not desired, NC may be stored in TBST overnight at 4 °C.

Alternatively, a semi-dry protein transfer can be performed. Follow the instructions in the manual for the Trans-Blot® SD Semi-Dry Electrophoretic Transfer Cell. **Towbin buffer** (25 mM Tris, 192 mM glycine, 20% methanol) works well as a transfer buffer in this system. Do not adjust the pH of the buffer. To transfer proteins from 2 mini-gels simultaneously, set the voltage to 15 V and the current limit at 0.88 A and transfer for 30 min. The advantage of the semi-dry system is speed.

WESTERN BLOTTING (IMMUNOBLOTTING)

Equipment and materials

- automatic film developer (Kodak X-OMAT M43A Film Processor or Bio-Rad Chemi Doc® 1000/2000 gel documentation system and Quantity One® quantitation software, Version 4 (Bio-Rad, Hercules, California)
- autoradiography film (Hyperfilm™ ECL™ high performance chemiluminescence film, Amersham Life Science RPN2114H)
- micropipetters (2 µL ,10µL, 1000 µL) and tips
- plastic wrap
- powder-free gloves
- small plastic trays to hold NC membranes (pipet tip tray lids)
- laboratory shaker
- nitrocellulose membrane from protein transfer procedure
- pH meter and calibration buffers
- stir plate and stir bars
- automatic pipetter and pipets (25 mL and 10 mL), or graduated cylinders
- small glass beakers
- paper towels or Kimwipes

Chemicals and reagents

- polyclonal rabbit anti-vitellogenin antiserum (primary antiserum, Michigan State University)
- biotinylated donkey anti-rabbit IgG, whole antibody (secondary antibody, Amersham Pharmacia Biotech RPN1004)
- streptavidin-peroxidase (tertiary antibody, Amersham Pharmacia Biotech RPN1231)
- ECL™ Western blotting reagents (Amersham Pharmacia Biotech RPN2109)
- Tris (Gibco 15504-012)
- sodium chloride (NaCl, Baker 3624-05)
- Tween-20 (Sigma P-7949)
- hydrochloric acid (HCl, for pH adjustment)
- Milli-Q water
- nonfat dry milk

Procedure

All of the following steps take place at room temperature.

Blocking

If the NC membrane was not blocked overnight, incubate on a shaker in 100 mL blocking buffer per membrane for 1-2 hr at room temperature.

Primary antisterum

Rabbit anti-vitellogenin antiserum developed at Michigan State University. Dilute primary antiserum 1:20,000 in TBST or blocking buffer. It takes about 40 mL of diluted antiserum to effectively cover a membrane cut to fit the gels used in this protocol (2 µL antiserum to 40 mL TBST). Incubate on a shaker (approximately 30 rpm) for 2 hr.

Rinse

Wash NC with TBST. Quickly rinse 3 times with 20 mL TBST per rinse. Then perform 3 rinses, each with 20-40 mL of fresh TBST for 10 min per rinse. The last three rinses should be performed on the shaker at a low setting.

Secondary antibody

Biotinylated donkey anti-rabbit IgG, whole antibody. Dilute secondary antibody 5 µL:10 mL TBST. Prepare 40 mL per NC (20 µL diluted in 40 mL TBST for one NC). Incubate for 1 hr on the shaker.

Rinse

Repeat as described above.

Tertiary antibody

Streptavidin-peroxidase. Dilute tertiary antibody 5 µL:10 mL TBST. Prepare 40 mL per NC. Incubate 20 min on the shaker.

Rinse

Repeat as described above.

Addition of ECL™ Western blotting reagents

Follow directions on the package. Briefly, mix 2 mL of Reagent 1 with 2 mL of Reagent 2 for one NC. Lay the NC on a tray and apply reagents. Follow incubation times listed in the instructions. Blot the edge of the membrane on a paper towel or Kimwipe, then lay the NC membrane face-down on plastic wrap. (Saran Wrap™ works well because it does not wrinkle as easily as some other brands of plastic wrap). Fold the plastic wrap so that none of the liquid can drip out. Turn the wrapped NC over and gently smooth out any air bubbles between the plastic wrap and NC. Do not attempt to reposition the NC; this could dislodge the proteins.

Luminescence detection

Expose NC to autoradiography film to detect luminescence and use an automatic film developer if available. The film processor should be turned on and allowed to warm up for 10 min prior to use. Alternatively, use the Bio-Rad ChemiDoc imaging system and Quantity One software to store an electronic image of the NC. The results are often better with the ChemiDoc than with film because the response is linear and minor bands do not tend to show up as they do with film.

TIPS:
Many types of autoradiography film can be used, but different types are optimized for certain applications. If high background tends to be a problem, pre-flash film can be used. High intensity red light is used to pre-expose the entire film prior to exposure to the NC. Hyperfilm™ ECL™ high performance chemiluminescence film (described above) works well, as it is optimized for use with the ECL western blotting detection system. Store film in a refrigerator if possible.

Use powder-free gloves during the Western blotting process. Powder from gloves can affect the NC and film.

<u>Using the imaging system:</u> Turn on the camera and light box. Open the program Quantity One and go to "Chemi-Doc" on the pull-down menu. Check that "lumi" is checked, and not "UV" or "white." Make sure the amber filter is not on the camera lens (it screws on and off from inside the light box). Turn on the epi-light and click "Live Focus" to determine where the NC should be placed. Turn the top lens (aperture) to one of the higher numbers at this point until the NC can be seen. It should be placed so that the outer edge is inside the outer red rectangle on the viewing screen. Place a card with writing on it over the NC to focus and zoom. Focus and zoom lenses are on the

camera above the light box. Twist them around to set the focus and zoom. Click "Freeze", remove the card, and shut the light box door. Shut off the epi-light and turn the top lens to 1 (opening the aperture). Type in the length of exposure time (generally 60 to 300 seconds), and press "Manual Exposure." The bar at the bottom indicates the time left during the exposure. The resulting photo will show up automatically at the end of the exposure. There are functions to remove background and to bring all of the bands out relative to the maximum density band. Save and print before attempting another exposure.

GEL STAINING

Equipment and materials

- laboratory shaker
- plastic or glass trays to hold gel
- laboratory gloves
- plastic wrap (to cover trays)

Chemicals

- methanol (J.T. Baker 9093-03)
- Coomassie Brilliant Blue R-250 (Gibco 15528-011)
- acetic acid (EM Science, Gibbstown, New Jersey)
- Milli-Q water

Procedure

After the protein has been transferred to the NC, the gel can be stored in water or TBST if there is some doubt that all of the protein transferred and another attempt might be made. If the intent is to stain the gel to look for remaining protein, place the gel in **destaining solution**, overnight if necessary. Cover the tray with plastic wrap and leave at room temperature. The acetic acid precipitates and fixes the proteins. If the gel is left in water or transfer buffer, the bands will begin to widen and blur. Follow the procedure for Coomassie Blue staining of gels described by Sasse and Gallagher (1991).

REAGENTS AND SOLUTIONS

10% SDS

SDS	50 g
water	to 500 mL

Store at room temperature indefinitely. Discard only if precipitate forms.

4X Stacking Gel Buffer

(0.5 M Tris, pH 6.8)

Tris	3 g
=> Adjust to pH 6.8 with HCl	
water	to 50 mL

Store at 4 °C, 6-12 months maximum.

2X Treatment Buffer

(125 mM Tris, 4% SDS, 20% glycerol, 10% 2-mercaptoethanol, pH 6.8)

Tris	2.5 mL 4X stacking gel buffer
SDS	4.0 mL 10% SDS
glycerol	2.0 mL
water	to 9.0 mL
bromophenol blue	2 mg (roughly 2 mm in the end of a Pasteur pipette)

Divide into aliquots and store in conventional freezer no longer than 6 months. Add 10% 2-mercaptoethanol (BME, β-mercaptoethanol) immediately prior to use. BME is highly toxic and smells foul, so work under a fume hood. The amount of bromophenol blue added to this buffer should result in a deep blue color.

10X Tank Buffer

Tris	30.28 g
glycine	144.13 g
water	to 1.0 L

It is not necessary to check the pH of this solution. It can be stored for over a year at room temperature.

Tank Buffer
(25 mM Tris, 192 mM glycine, 0.1% SDS, pH 8.3)

10X tank buffer	100 mL
10% SDS	10 mL
water	to 1.0 L

It is not necessary to check the pH of this buffer. Store at 4 °C for less than 3 months.

Transfer Buffer
(25 mM Tris, 192 mM glycine, 0.1% SDS, 20% methanol)

10X tank buffer	200 mL
methanol (MeOH)	400 mL
10% SDS	20 mL
water	to 2.0 L

Chill to 4 °C.

The MeOH tends to inhibit transfer of proteins, but it also keeps the buffer cool during protein transfer. If higher voltages are used (70-100 V) for transfer, use MeOH in the buffer. If lower voltages are used (approx. 40 V), MeOH can be left out but is still recommended.

Towbin Buffer
(25 mM Tris, 192 mM glycine, 20% MeOH)

Tris	3.03 g
glycine	14.41 g
MeOH	200 mL
water	to 1.0 L

Important: Do not adjust pH.
Chill to 4 °C.

Also, 0.1% SDS (10 mL 10% SDS) may be added if desired. To optimize transfer of large proteins (>150 kD), concentration of MeOH may be reduced.

10X TBS (Tris-Buffered Saline)
(200 mM Tris, 1.54 M (9%) NaCl, pH 7.5)

Tris	24.22 g
NaCl	90.0 g
=> Adjust to pH 7.5 with HCl	
water	to 1.0 L

Store at 4 °C for 6-12 months.

TBST (Tris-Buffered Saline and Tween-20)
(20 mM Tris, 154 mM (0.9%) NaCl, 0.1% Tween-20, pH 7.5)

10X TBS	100 mL
Tween-20	1.0 mL
=> Adjust to pH 7.5 if necessary	
water	to 1.0 L

Store at 4 °C for less than 3 months. Some protocols use 100 mM Tris.

Blocking Buffer
(3% (w/v) nonfat dry milk in TBST)

nonfat dry milk	3.0 g
TBST	to 100 mL

Mix immediately prior to use. Up to 5% nonfat dry milk is typically used.

Ponceau S Staining Solution
(0.1% (w/v) Ponceau S, 5% acetic acid)

Ponceau S	1.0 g
acetic acid	50 mL
water	to 1.0 L

Coomassie Blue Staining Solution
(50% MeOH, 0.05% (v/v) Coomassie Brilliant Blue R-250, 10% (v/v) acetic acid)

methanol	250 mL
Coomassie Brilliant Blue	0.25 g
acetic acid	50 mL
water	to 500 mL

Store at room temperature for up to 6 months. Can be reused but staining efficiency will decrease with each use. If precipitate forms, filter to obtain a homogeneous solution.

Destaining Solution
(5% MeOH, 7% acetic acid)

methanol	50 mL
acetic acid	70 mL
water	to 1.0 L

Store at room temperature for less than 1 month.

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