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Viability of Male Gametes in Common Carp (*Cyprinus carpio*) along the Lower Colorado River from the Cibola National Wildlife Refuge (NWR), Havasu NWR, and Lake Mohave of Lake Mead National Recreation Area

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Outside front cover photograph: Cibola NWR. The site is a razorback sucker and bonytail refugium.

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Abstract

To contribute to an investigation on possible endocrine impacts in three sites along the lower Colorado River in Arizona, especially in male fishes, this study addressed the null hypothesis that aquatic species in southern sites did not exhibit evidence of endocrine disruption as compared with those in nonimpacted sites. The results presented are intended to provide managers with science-based information and interpretations about the reproductive condition of biota in their habitat along the lower Colorado River to minimize any potential adverse effects to trust fish and wildlife resources and to identify water resources of acceptable quality. In particular, these data can inform decision making about wastewater discharges into the Colorado River that directly supplies water to Arizona refuges located along the river. These data are integral to the USFWS proposal entitled "AZ – Endocrine Disruption in Razorback Sucker and Common Carp on National Wildlife Refuges along the Lower Colorado River" that was proposed to assess evidence of endocrine disruption in carp and razorback suckers downstream of Hoover Dam.

Background Information

Because biota in aquatic ecosystems are continuously exposed to constituents over their entire body surface, instances of hormonal disturbance are not unexpected. Because of this exposure, constant low levels of potent estrogenic compounds, like ethynyl estradiol, can result in significant endocrine and reproductive effects in aquatic biota (Sumpter and Johnson, 2005). Waters containing endocrine disrupting compounds and their effects on aquatic biota have been demonstrated in the lower Colorado River in Arizona and Nevada (Bevans and others, 1996; Goodbred and others, 1997; Snyder and others, 1999; Snyder and others, 2001; Patino and others, 2003). In light of this, information based on scientific studies would help inform managers on critical decisions being considered for using water from such sources to provide more inflow to some of Arizona's national wildlife refuges (NWR). The Arizona refuges along the lower Colorado River provide habitat to the endangered razorback sucker (*Xyrauchen texanus*) and the bonytail chub (*Gila elegans),* as well as endangered avian species, such as the willow flycatcher (*Empidonax traillii*) and the Yuma clapper rail (*Rallus longirostris yumanensis*).

Many human-made chemicals can disrupt endocrine systems of wildlife (Colborn and Clement, 1992) and can affect tissues at concentrations well below detectable levels. Hence, for investigating causal effects, the use of biomarkers for assessing contaminant exposure is necessary (Carey and Bryant, 1995). Environmental toxicants known as endocrine disruptors may have estrogenic, antiestrogenic, thyroid-

disrupting, androgenic, or antiandrogenic effects that may impair important processes of the endocrine system like metabolism, reproduction, and sexual developmental (Carey and Bryant, 1995). Most endocrine-disrupting studies have focused on reproductive impacts; however, other effects have been documented, including those on growth, metabolism, thyroid, and immune function (Gross and others, 2003). In fact, in male mammals, damage to fertility caused by transient exposure of embryos to endocrine-disrupting environmental toxins can be passed down to subsequent generations (Anway and others, 2005). Several fish species have been used for in vivo, multiple generational testing of endocrine disrupting compounds, including Japanese medaka (*Oryzias latipes*) (Patyna and others, 1999), zebrafish (*Pterois volitans*) (Nash and others, 2004), fathead minnow (*Pimephales promelas*) (Lange and others, 2001), and mummichog (*Fundulus heteroclitus*) (Bordeau and others, 2004).

To contribute to an investigation on possible endocrine impacts in three sites along the lower Colorado River in Arizona, especially in male fishes, this study addressed the null hypothesis that aquatic species in southern sites did not exhibit evidence of endocrine disruption as compared with those in nonimpacted sites. The results presented are intended to provide managers with science-based information and interpretations about the reproductive condition of biota in their habitat along the lower Colorado River to minimize any potential adverse effects to trust fish and wildlife resources and to identify water resources of acceptable quality. In particular, these data can inform decision making about wastewater discharges into the Colorado River that directly supplies water to Arizona refuges located along the river. These data are integral to the USFWS proposal entitled "AZ – Endocrine Disruption in Razorback Sucker and Common Carp on National Wildlife Refuges along the Lower Colorado River" that was proposed to assess evidence of endocrine disruption in carp and razorback suckers downstream of Hoover Dam.

Introduction

Bioindicators are biomarkers and biocriteria which are measurable and directly reflect the condition of the animal or the natural resources, respectively. Two important features of animal biomarkers are the ability to detect interactions between stressors and organisms and the capability of quantifying sublethal effects. Using multiple lines of evidence in an investigation and more than one biomarker can provide a better assessment of the physiological effects and bioavailability of xenobiotic compounds found in aquatic habitats. This report highlights studies on sperm cell viability in male carp from Havasu and Cibola NWRs where evidence of contaminant impacts has been documented (Andrews and others, 1997), and from Lake Mohave, which is part of the U.S. Park Service's Lake Mead National Recreation Area. Lake Mohave is upstream from some sources of contaminants; however, it is below Lake Mead, where

evidence has demonstrated xenobiotic effects on reproduction in carp (Bevans and others, 1996; Patino and others, 2003). Also included are data on fish lengths and weights and the gonadosomatic index (GSI) in order to facilitate the integration of all data from each study component. The larger project is being managed by Carrie Marr, Wildlife Biologist, Environmental Contaminants, USFWS, Region 2, Phoenix Arizona Ecological Services Field Office.

When examining feral populations of fish, considerable variation can occur in nutrition and reproductive status (Bevans and others, 1996), so it is important to collect a large enough sample size to adequately represent the population at study sites. Extracellular receptors in fish involved in nutrient uptake can be altered by exposure to xenobiotics (Shlenk, 2001). Xenobiotic pollutants may also disrupt reproductive endocrine function by acting on a variety of organs, such as along the hypothalamus-pituitary-gonadal axis, as well as within the communication systems between endocrine and immune systems (Arcand-Hoy and Benson, 2001). Disruption at any of these sites may result in either changes in the rate of gonadal development or in the viability of the gametes, as well as the arrest of gametogenesis or decreased GSI (Kime and Nash, 1999). Inadequate nutrition can have as great an effect on GSI, egg production (fecundity), and basal metabolism, as can exposure to xenobiotics (Patyna and others, 1999). This report provides results on the viability of male gametes and on morphological measures of carp, including fish length, weight, and gonadosomatic indices.

A sperm cell consists of several membrane compartments, and cell competency requires that each of these membrane compartments be intact (Graham and others, 1990). Sperm plasma membrane organization, fluidity, permeability, and lipid composition are parameters important to membrane integrity. The classic method of assessing the viability of sperm is to determine the percentage of progressively motile cells by using a microscope (Jenkins, 2000). This is an indirect method of assessing metabolic activity, and results reflect inherent variability because of the subjective nature of collecting data on motility by individual researchers using microscopy. In this study, sperm membrane integrity was assessed by a cell viability stain, where nucleic acids inside intact membranes were stained fluorescent green by SYBR-14, whereas nonintact or permeable membranes allowed a red counterstain (propidium iodide) to enter the cells so that "dead" cells stained fluorescent red. Upon cell death, propidium iodide (PI) rapidly overwhelms the fluorescence exhibited by SYBR-14. This fluorometric staining combination of SYBR-14 and PI has been shown to be a rapid and reliable means for determining the proportions of living and dead sperm in several species across taxonomic lines (Ericsson and others, 1990; McNiven and others, 1992; Garner and Johnson, 1995; Donoghue and Donoghue, 1997; Thomas and others, 1998; Segovia and others, 2000; Adams and others, 2003; Lezcano and others, 2004; Salinas and others, 2005).

Xenobiotic exposure of fish in aquatic ecosystems can affect sperm quality and reproduction in many different ways. A common reaction that occurs between certain xenobiotics and lipids is peroxidation which can produce toxic intermediates affecting many different physiological processes including significantly altered membrane integrity in sperm cells (Schlenk, 2001). Sperm cell integrity has been interrupted by membrane peroxidation in turkey (*Melleagris gallopavo*) (Donoghue and Donoghue, 1997) whereby reactive oxygen species induce tissue damage and motility can be lowered. Besides altering sperm viability, xenobiotics can also cause reduced sperm counts (Haubruge and others, 2000), reduced GSI (Jobling and others, 1996), altered sexual behavior (Bayley and others, 2002), intersex gonads (Metcalfe and others, 2001), decreased secondary sex characteristics (Harries and others, 2000), and delayed sexual maturation (Segner and others, 2003). If exposure of xenobiotics that are potent endocrine-disrupting compounds (EDCs), like ethynylestradiol (EE2), is long enough even at very low levels, they can cause reproductive failure in fish (Nash and others, 2004). Since there are many different types of xenobiotics present in aquatic ecosystems that may affect sperm quality in different ways, a combination of several sperm quality assays is the best way to assess fertility in male fish (Jenkins, 2000). Reductions in reproductive biomarkers, caused by whatever source and through whichever mechanisms, can be reflective of exposure to xenobiotics; if significant enough, they can ultimately impact fish populations.

Materials and Methods

Collections

Male common carp were obtained by the U.S. Fish and Wildlife Service from Cibola and Havasu NWR (southern sites) and from Lake Mohave (northernmost site) (fig. 1) along the lower Colorado River in Arizona. Fish (n = 11) were collected from the northernmost site (Lake Mohave, and from two southern sites, Cibola NWR (n = 2) and Havasu NWR (n = 8). Fish were processed, and samples were taken for measuring a variety of reproductive biomarkers.

Morphological measures of fish included fish total length, weight, and gonad weight. These data were employed in statistical analyses to determine difference in biomarkers between sites. Testes were dissected from each mature male fish (fig. 2) and were sent overnight to U.S. Geological Survey's National Wetlands Research Center (NWRC) in a modified Hanks' buffered salt solution (HBSS), pH 7.4 without calcium, at 304 mOsm/kg (Glenn, 1998). Osmolarity of milt and buffers were measured by vapor pressure osmometry (Wescor, Logan, Utah).

Figure 1. Carp were collected from Cibola and Havasu national wildlife refuges, and Lake Mohave.

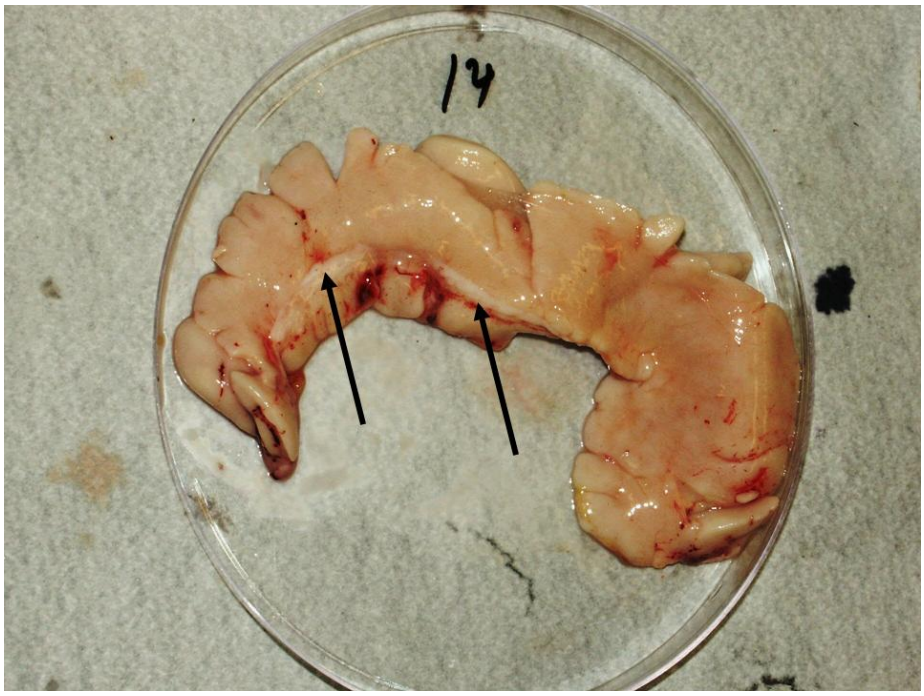

Figure 2. Carp testis (#13) collected from Havasu National Wildlife Refuge. Arrows indicate collectable milt.

Preliminary Experiments at NWRC

Dissection methods, buffer choice, and sperm cell preparation methods were optimized specifically for common carp. For sperm viability and motility analyses, extracted testes were shipped overnight in HBSS with 10% v/v streptomycin/penicillin. Milt (a suspension of sperm and seminal fluid) was sampled from the posterior portion of testes (fig. 2), and the proportion of living cells was assessed with flow cytometry (see below).

Flow Cytometry

Flow cytometry is powerful technology for studying cells. Individual cells are probed by a laser light, and emitted light signals are transferred quickly into electronic storage so that typical sample sizes approximate 10K – 50K cells that are collected within ~1 min. In the membrane integrity staining used in this study, SYBR-14 stained the nucleic acids in nuclei of living sperm, and the counterstain propidium iodide stained dead or moribund sperm exhibiting both fluorescent colors whose membranes were not intact. Sperm viability assays by flow cytometry assess the functional capacity of sperm in an objective manner. Gamete viability is a direct measure of the function of cells involved in reproduction.

Sperm Cell Viability

In order to validate the sperm viability assay, individual *Cyprinus carpio* common carp (n = 3) were obtained locally, and standard curves were generated by using known live and dead cell mixtures. A known concentration of sperm at 1 x 10^6 per mL (3 mL in a 10 mL tube) was killed by heating at 80° C in a waterbath for 10 min. The assay was verified by identifying viable and nonviable cells of known mixtures of untreated and heat-killed sperm by using five replicates per fish for three individuals (fig. 3).

Sperm suspensions were filtered with 30 Fm nylon mesh (Small Parts, Miami Lakes, Fla.) prior to dilution to 1 x 10^6 /mL. Cells were stained by using a live/dead sperm viability kit (Molecular Probes, Eugene, Oreg.) with a starting stock solution of 1:100 SYBR-14 aliquoted into 250 µl cell suspensions (Segovia and others, 2000). A flow cytometer (FACScan, Becton Dickinson Immunocytometry System, San Jose, Calif.) was used to analyze the sperm. This multiparameter analytical technique can be used for assessing nuclear and cellular components of interest in freely flowing cells. For verification of cell condition and probes employed in flow cytometry, epifluorescence microscopy (Leitz Diaplan) was used. Per sample, 10,000 cells were analyzed in triplicate.

Statistics

After testing for statistical differences between the Cibola and Havasu sperm viability data and finding none, data were combined for these southern sites (n = 10). The alpha level was set at < 0.05. Sperm viability proportions and GSI data were arcsin[sqrt] transformed to obtain normality. Correlation analyses were performed to investigate if fish lengths and weights were covariates with sperm viabilities and GSI. A one-way ANOVA was used to test for differences in sperm viabilities and morphological parameters between northernmost and southern sites. Discriminant analysis was performed to determine rates of misclassification of gonad to body weight ratio data between sites. A t-test was used to test for differences in fish lengths and fish weights between sites.

Results

For three individuals, the sperm viability assay was verified (fig. 3) in triplicate. The R^2 values for each curve per fish were 0.9911 , 0.9867, and 0.9951, thereby validating the assay.

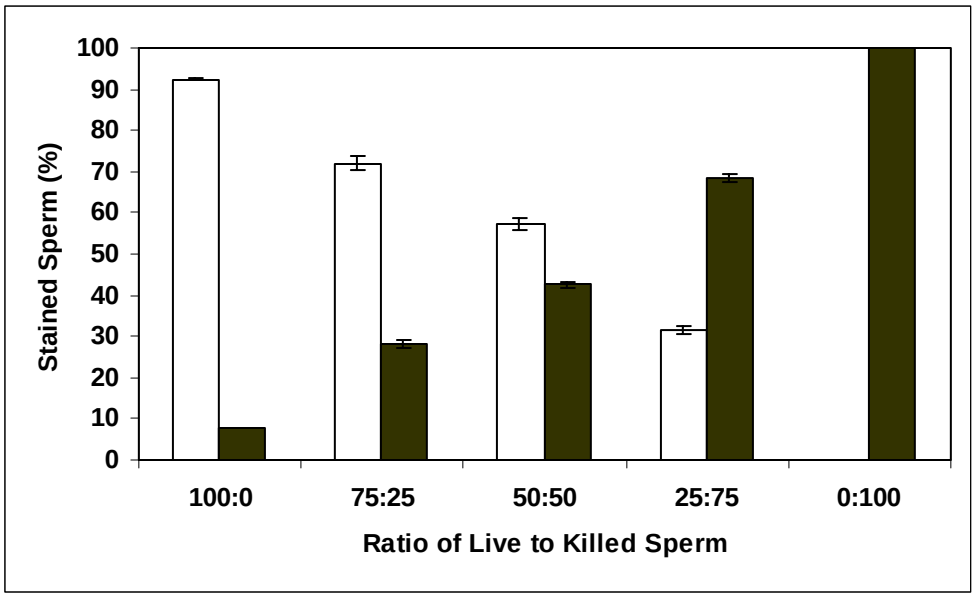

Figure 3. With a carp of starting viability of 92.3% ± 0.2 (SE), flow cytometric measures were made of known live and dead cell mixtures.

No significant correlations were found for fish weights and lengths with sperm viability proportions. Duncan's Multiple Range test showed no significant differences between Cibola and Havasu, so pooling data was justified. A significant difference was noted in sperm viabilities between northernmost and southern sites, with $F_{(1,41)} = 29.60$, $P<0.0001$ for this two site analysis (fig. 4).

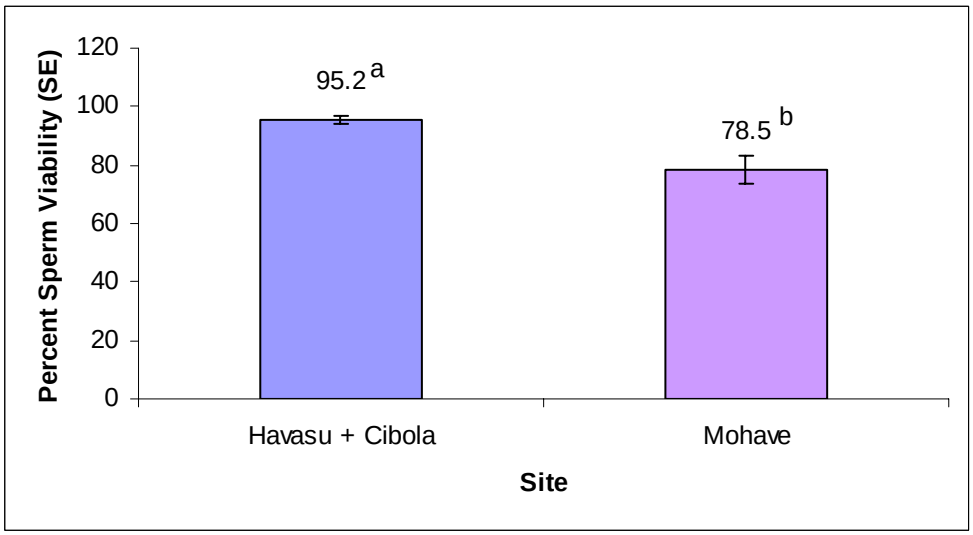

Figure 4. Mean sperm viability (±SE) from carp collected from southern sites (Havasu and Cibola) were significantly higher than those from the northernmost site (Mohave) ($P<0.0001$).

Length and weight were not correlated with gonad to body weight ratios, so were not used as covariates in the model. The results of the one-way ANOVA showed a significant difference between the sites ($F_{(1,41)} = 57.28$, $P<0.0001$) (fig. 5).

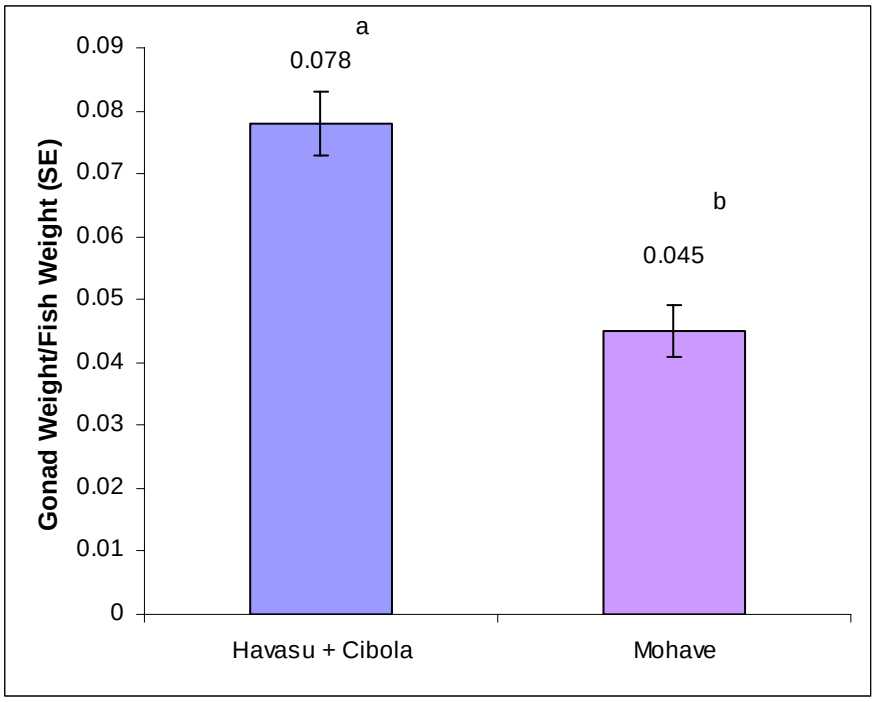

Figure 5. Mean gonad to body weight proportions (±SE) show that values from carp collected from southern sites (Havasu and Cibola) were significantly higher than those from the northernmost site (Mohave) (P<0.0001).

When a discriminant analysis using arcsin(sqrt) gonad to body weight ratio = arcsin(sqrt[testis weight/total weight]) was performed to obtain the rates of misclassification of carp into the appropriate sites, Mohave was misclassified 8.7% of the time, and the southern sites were misclassified 10.0% of the time, indicating low misclassification when using gonad to body weight ratios.

Significant differences were noted in fish lengths (P < 0.0286) (fig. 6) and weights (P < 0.0126) (fig. 7) between sites, with higher values at the southern sites versus the northernmost site.

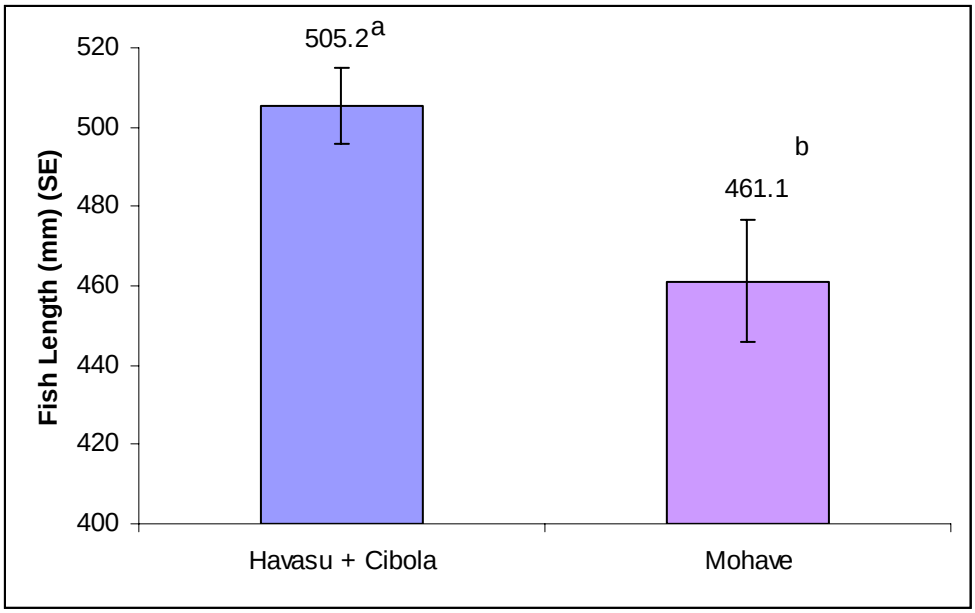

Figure 6. Whole body lengths (± SE) of carp collected from sampling sites.

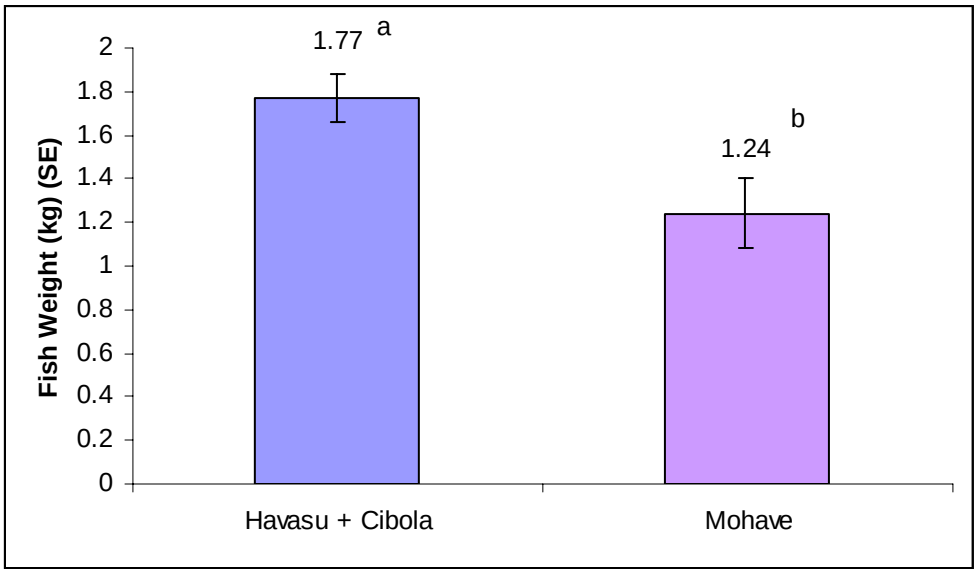

Figure 7. Whole body weights (± SE) of carp collected from sampling sites.

Discussion

Current Study

The focus of this study was to investigate possible reproductive impacts in carp from waters of Arizona National Wildlife Refuges along the lower Colorado River. The source of water for these Arizona refuges is Lake Mead formed by Hoover Dam, where reproductive effects in fish have been documented (Bevans and others, 1996; Goodbred and others, 1997; Patino and others, 2003). The data on common carp from this study are being compiled with data collected from endangered razorback suckers from the same region (fig. 1). This study was performed as a basis for science-based management of wastewaters (see Project ID: 1261-2N27, Environmental Contaminants Program, On-Refuge Investigations Sub-Activity, Region 2). Results from this study are being incorporated as part of this larger investigation. Results from this study show significantly lower sperm viability and GSI in male carp from Lake Mohave below Lake Mead compared to that in the carp from Cibola and Havasu NWRs. This lower viability may be due to increased exposure to xenobiotics and EDCs or possibly to differences in nutrition or water temperature. Results from contaminant analysis in fish tissue and bed sediment will help assess differences in exposure between the sites. It has already been discussed that fish in Lake Mead above Lake Mohave have been documented to have significant evidence of endocrine and reproductive effects (Bevans and others, 1996; Patino and others, 2003). Transport of the xenobotics that might have caused these effects in Lake Mead could be expected to flow downstream into Lake Mohave, especially if they are hydrophilic (Kolpin and others, 2002). Reduced sperm viability effectively reduces the ability of

that male fish to fertilize eggs. Ratios of 1,500 sperm per egg are needed for good fertilization rates in the catfish *Clarias batrachus* (Rurangwa and others, 1998). It has been suggested that male fish in the wild closely control the sperm/egg ratio to achieve the minimum for full fertilization (Warner, 1997). If this same ratio is maintained by using milt from a fish that has 18% reduced sperm viability similar to fish from Lake Havasu, however, the resulting ratio of 1,230 viable sperm per egg would likely result in a reduced fertilization rate.

Of equal or more concern is the 42% reduction in GSI in fish from Lake Mohave. Reduced GSI has been shown in male fish exposed to the natural female steroidal hormones estradiol (Kang and others, 2002), estrone and estriol (Metcalfe and others, 2001), the synthetic steroid hormone EE2 (Jobling and others, 1996), biodegradates of detergents like nonylphenol and octylphenol (Nimrod and Benson, 1998), and pentylphenol (Gimeno and others, 1998), and bisphenol A used in epoxy resins (Sohoni and other, 2001). Clearly, a reduced GSI lowers an individual male's ability to fertilize eggs, and if decreased significantly enough, it could lower recruitment and population levels. It is important to keep in mind, however, that this study only sampled once during the reproductive year, and the sample size is relatively small. Further studies would need to be done to better assess if reduced GSI in male fish from Lake Mohave are seen thoroughout the year and in different species including the endangered razorback sucker. Also population modeling would need to be done in Lake Mohave to determine if any of these altered reproductive biomarkers might result in reduced population levels.

Reproductive and Endocrine Toxicology

Sex Steroid Hormones

Sex steroid hormones are produced by the endocrine system in animals and control important stages of the life cycle of an organism including gametogenesis, fertilization, sexual development, and reproduction (Gross and others, 2003). Research in the past decade has established that a wide variety of anthropogenic compounds in the environment are capable of modulating and adversely affecting or disrupting endocrine function in animals (Tyler and others, 1998). Molecular binding processes are involved with maintaining hormonal balance, and alterations to the mechanistic proteins or types of estrogenic compounds can influence ligand/receptor interactions and consequent transcription. Teleost fish have three forms of estrogen receptors with positions of conserved residues important in binding estrogen and estrogen mimics (Hawkins and Thomas, 2004). A specific sex steroid-binding protein involved in regulation of circulating sex steroids, steroid delivery to target cells, and intracellular signaling in sex steroid-sensitive tissues has affinity for estrogen mimics and hence may serve as another pathway for endocrine disruption (Tollefsen and others, 2004). Quite a few compounds have been found to bind with the estrogen receptor (Vonier and others, 1996), but the synthetic steroid ethynyl estradiol used in oral contraceptives is by far the most potent xenoestrogen (Sumpter and Johnson, 2005). Genistein, a primary isoflavone from soybeans and a phytoestrogen, can bind to estrogen receptors and has been shown to enhance testicular development in rainbow trout, yet sperm motility and concentration were decreased in a dose-dependent manner at spawning (Bennetau-Pelissero and others, 2001). Over 65 different types of chemicals have been identified as being endocrine disruptors (Keith, 1997), including PAHs, PCBs, dioxins and furans, several trace elements, organochlorine, organophosphate, and carbamate pesticides, triazine herbicides, and synthetic steroids (Gross and others, 2003).

Gametogenesis

There is still a poor understanding about the basic mechanisms regulating the genetic control of male fertility in mammals. Products from over 3,000 genes have been found to be involved in the circuits regulating the expression of male and female fertility (Vogt, 2005). In humans, it is estimated that male infertility has a genetic basis in 30% of men (McLachlan and others, 1998). Male germ cell differentiation requires highly regulatory processes and signaling cascades that contribute to spermatogenesis (Krausz and Sassone-Corsi, 2005). Advances in how particular genes regulate key events, such as the way by

which DAZ genes regulate metazoan gametogenesis (Reynolds and Cooke, 2005), and determining whether there are environmental impacts on gene expression, are good areas for future investigation.

Gametogenesis is largely determined by the control of estrogens and androgens, with estrogens also playing a role in males. The predominant androgen necessary for spermatogenesis in lower vertebrates is 11-ketotestosterone, and concentrations have been shown to correlate with testicular development and spermiation in black porgy (*Acanthopagrus schlegelii schlegelii*) (Chang and others, 1995). Low concentrations (10 ng/L) of estrogen or synthetic estrogen have been found to stimulate spermatogonial proliferation rather than higher concentrations (100 and 1,000 ng/L) (Song and Gutzeit, 2003). Several disorders of sexual development in humans have been attributed to disruption of the androgen receptor gene located on the X chromosome (McLachlan and others, 1998). Antiandrogens have been shown to decrease sperm counts. Some studies have investigated spermatogenesis, such as with Japanese medaka testes in culture (Hong and others, 2004), in minimizing side effects in mammals by chemotherapeutic agents (Jyothi and others, 2001), and in detecting testicular toxicity (Yoon and others, 2003).

Spermatogenesis

The testis of carp contains an organized system of lobules that radiate perpendicularly from a central longitudinal duct. Tubule lumens are filed with spermatozoa in mature males (Patino and others, 2003). Spermatogenesis is a process of cell differentiation characterized by mitotic and meiotic divisions that transform the spermatogonia (male germ cells that produce cells for the next generation) into final mature spermatids. By spermiogenesis, the spermatids elongate and transform into spermatozoa. The animal cell cycle consists of a round of chromosomal DNA replication in S-phase followed by segregation of the replicated chromosomes into the daughter nuclei. Meiosis, the cell division occurring in germ cells, is a specialized part of the cell cycle that consists of a round of DNA replication followed by two successive rounds of chromosome segregation. Meiosis produces progeny cells with half as many chromosomes as their parent cells, thus making sexual reproduction possible (Dekel, 1995). Flow cytometry has become a useful tool for objective quantification of the types of testicular cells involved in spermatogenesis (Yoon and others, 2003). Forms detectable include haploid spermatids (the most mature form of sperm cell types), diploid cells (spermatogonia, secondary spermatocytes, and tissue somatic cells), and tetraploid cells (mostly primary spermatocytes) (Yoon and others, 2003). When damage to sperm occurs at later stages of spermiogenesis in the testis or in the epididymal sperm, sperm numbers may be normal, but sperm viability or motion characteristics, and therefore sperm function, may be impaired (Perreault, 1998).

Many studies have shown that xenobiotic compounds that act as endocrine disruptors can inhibit the development of sperm maturation in fish (Kime, 1998; Gimeno and others, 1998) and in other animals (Staub and others, 2002). Analysis of ploidy levels of sperm in a milt sample allows for determining the relative proportion of sperm that are haploid or "mature," with the more reproductively ripe individuals showing higher numbers of spermatids. By using flow cytometry and DNA staining of sperm cells, these cell types with different amounts of DNA can be easily enumerated.

Reproduction

Because fish are in direct contact with the aqueous environment, exposure to compounds in water occurs over the entire body surface (Schlenk, 2001). Fish reproduction is one of the most sensitive indicators of exposure to sublethal concentrations of environmental chemicals (Arcand-Hoy and Benson, 2001). Effects of environmental stressors on reproduction ultimately affect population levels of biota (Donaldson, 1990), but conducting studies at this level is expensive and time consuming. Although laboratory data support the hypothesis that EDCs can impact reproductive health of various fish species, little evidence from field studies has shown actual impacts to fish populations (Mills and Chichester, 2005). At least one study, however, has demonstrated a causal relationship between EDCs, reproductive impairment, and population level effects in American alligators (*Alligator mississippiensis*) (Guillette and others, 1994). Another field study where a lake was dosed with a low level of EE2 caused a significant decline in fathead minnow populations (Pelley, 2003).

The field of reproductive toxicology is widespread including both field and laboratory studies with several different fish species. Applications include basic screening tests which are relatively simple and less costly to detect hazards and in-depth tests to identify and elucidate mechanisms of toxicant action. In mammalian studies, integrity of sperm membranes under hypoosmotic conditions is the basis for a commonly used sperm quality test, the hypoosmotic swelling test. Generally, reduced sperm quantity or quality in animals can be caused by alterations in testicular development, intrinsic defects in the ability of germ cells to divide and differentiate, or defects or impacts on the hormonal regulatory pathway (McLachlan and others, 1998). In fish gamete biology, assessments of sperm quality include measures of concentration, aspects of motility, and fertilization ability (Belova, 1983). New technologies continue to be developed, and these are expanding our understanding of the basic biology of gametes and gamete function (Perreault, 1998).

Factors Influencing Reproductive and Endocrine Physiology

Contaminants and Stress

General animal health is a requirement for maintenance of proper physiological mechanisms and can be impacted by stress. The stress response of vertebrates involves several endocrine tissues and targets. Toxicants have been shown to act as stressors and interact with several endocrine processes (Quabius and others, 2000) including sperm quality (Gross and others, 2003). In all teleosts, cortisol produced in the renal cortex is the major corticosteroid produced under stress, and it has been indicated as the major factor that mediates the suppressive effects of stress on reproduction (Consten and others, 2002). Circulating levels of cortisol are often used as an indicator of the degree of stress experienced by fish (Adams, 2002). In studies of stress responsiveness of Mozambique tilapia (*Oreochromis mossambicus*) and rainbow trout (*Oncorhynchus mykiss*) (Quabius and others, 2000), poor nutritional status was shown to enhance the negative effects of polychlorinated biphenyl (PCB). The PCB concentrations were inversely proportional to sperm quality and quantity in men who had consumed PCB-laden fish (Rozati and others, 2000).

Nutrients

There are a number of ways that chemical substances can interfere with reproduction. Evidence has been shown for the existence of a blood-testes barrier (Schlenk, 2001). Consequently, distribution to such a sequestered tissue would be diminished unless the xenobiotic was highly lipophilic or could mimic endogenous ligands. Most gut-absorbed material is transported via the portal venous system to the liver, however, which is intimately connected with the hypothalamus-pituitary-gonadal axis (Arcand-Hoy and Benson, 2001), the site of synthesis of yolk protein, steroid hormones, and essential enzymes and cofactors; thus, the liver is generally considered to be a primary target for gut-absorbed chemicals (Schlenk, 2001). Many enzymes and receptors require cofactors such as metals or organic moieties (such as phorphyrins or folic acid). If cofactors are modified, then protein functions may be severely altered. If affected proteins are critical for cell maintenance, it is likely that cell viability may be ultimately compromised.

Gonadal development and fecundity are affected by certain essential dietary nutrients, especially in continuous spawners (Izquierdo and others, 2001). Lipid and fatty acid compositions have been identified as major dietary factors that determine successful reproduction and survival of offspring. The most common reaction that occurs between toxicants and lipids is lipid peroxidation (Schlenk, 2001). Dietary polyunsaturated fatty acids in sperm membrane phospholipids have been manipulated in sea bass

(*Dicentrarchus labrax*) (Bell and others, 1996) to influence sperm motility and fertility in domestic chickens (*Gallus domesticus*) (Cerolini and others, 2005), and sperm membrane integrity in rainbow trout (Pustowka and others, 2000). Highly unsaturated fatty acids with 20 or more carbon atoms, either directly or through their metabolites, affect fish maturation and steroidogenesis (Izquierdo and others, 2001).

Nutrients that add to the protection of reactive oxygen species contribute to the protection of membrane integrity, such as the selenium-dependent enzyme glutathione peroxidase (Wu and others, 1979). Storage of turkey semen has resulted in changes in phospholipid content caused by membrane phophoplipid lysis followed by endogenous metabolism or combinations of lysis, metabolism, and peroxidation (Douard and others, 2000). Antioxidants such as ascorbic acid (Dabrowski and Ciereszko, 2001), which are important in male trout because of their long-term effects during spermatogenesis (Ciereszko and Dabrowski, 2000), help maintain the genetic integrity of sperm by preventing oxidative damage to DNA (Dabrowski and Ciereszko, 1996). In mammals, the family of glutathione peroxidases comprises four mammalian selenoproteins (Brigelius-Flohe and others, 2003), whose functions include acting as a barrier against hydroperoxides derived from diets from metabolism of ingested xenobiotics. Vitamin E deficiency retards gonadal maturity and lowers hatching rate and survival of offspring (Izquierdo and others, 2001). Carnitine, a supplemental amino acid, has antioxidant properties that reduce the availability of membrane lipids for peroxidation in roosters (*Gallus domesticus*) (Neuman and others, 2002). In shrimp (*Litopenaeus vannamei*), dietary deficiencies were shown to reduce body weight gain and sperm count, yet there was no impact on abnormal sperm or animal survival (Perez-Valazquez and others, 2003). Sertoli cells in human testicular tissues have been found to be the most important cell type for metabolizing essential fatty acids (Retterstol and others, 2001). A closer investigation into this cell type may be another biological endpoint for investigation in endocrine disruption studies.

Particular micronutrients such as trace elements have been shown to impact sperm quality, especially from studies in nonmammalian animals. In boron-deficient male African clawed frogs (*Xenopus laevis*), a decrease in testis weight and sperm count was noted (Fort and others, 2002). In selenium-deficient rats (*Rattus* spp.), loss of male fertility was shown by sperm defects, particularly in mitochondrial-associated structures (Marin-Guzman and others, 2000) expressed during spermiogenesis and maturation (Olson and others, 2004). In zinc-deficient mice (*Mus musculus*), several impacts in testes were shown, such as reduction in sperm numbers, improper maturation of sperm, and decreased motility and fertility (Choudhary, 1995).

Conclusions

In conclusion, fish from Lake Mohave had significantly lower sperm quality and gonadal weights (GSI) than the fish from Cibola and Havasu NWRs. The alteration of both of these reproductive biomarkers clearly could reduce the fertilization ability of male fish from Lake Mohave and may be the results of exposure to xenobiotics and EDCs. Yet, since other factors like nutrition and temperature play a large role in reproductive conditions, further studies would be needed to assess if any such factors are involved in causing the altered reproductive biomarkers in male fish from Lake Mohave. In addition, future studies should also include additional sperm quality parameters, such as maturity and counts, which will assist in determining male reproductive condition and in assessing where in the reproductive process effects, if any, are occurring.

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