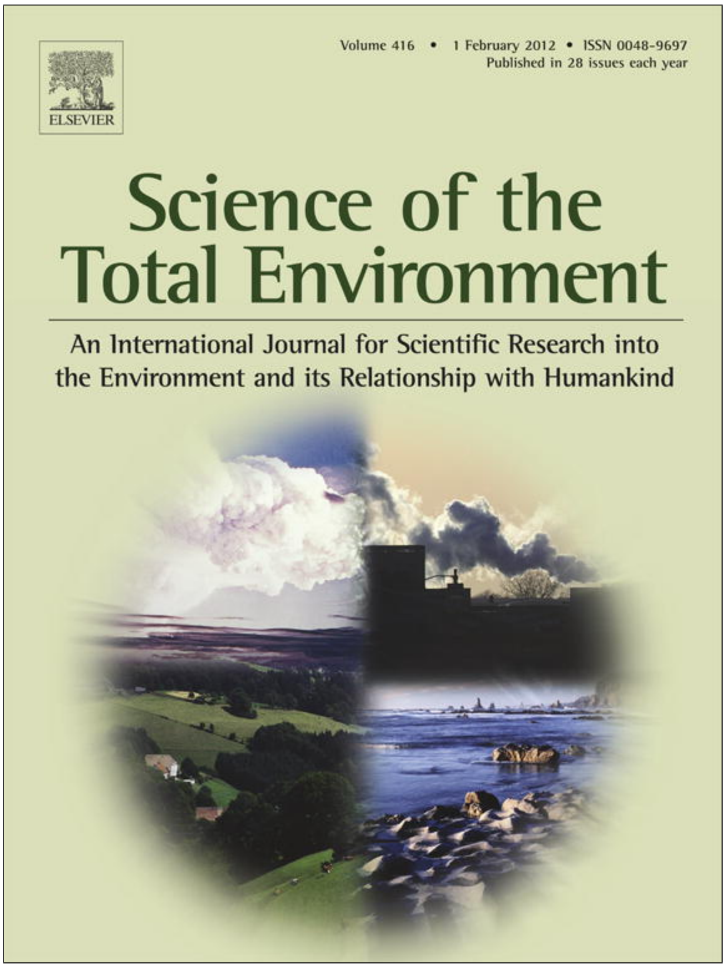
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Patterns of metal composition and biological condition and their association in male common carp across an environmental contaminant gradient in Lake Mead National Recreation Area, Nevada and Arizona, USA

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ABSTRACT

There is a contaminant gradient in Lake Mead National Recreation Area (LMNRA) that is partly driven by municipal and industrial runoff and wastewater inputs via Las Vegas Wash (LVW). Adult male common carp (*Cyprinus carpio*; 10 fish/site) were collected from LVW, Las Vegas Bay (receiving LVW flow), Overton Arm (OA, upstream reference), and Willow Beach (WB, downstream) in March 2008. Discriminant function analysis was used to describe differences in metal concentrations and biological condition of fish collected from the four study sites, and canonical correlation analysis was used to evaluate the association between metal and biological traits. Metal concentrations were determined in whole-body extracts. Of 63 metals screened, those initially used in the statistical analysis were Ag, As, Ba, Cd, Co, Fe, Hg, Pb, Se, Zn. Biological variables analyzed included total length (TL), Fulton's condition factor, gonadosomatic index (GSI), hematocrit (Hct), and plasma estradiol-17β and 11-ketotestosterone (11kt) concentrations. Analysis of metal composition and biological condition both yielded strong discrimination of fish by site (respective canonical model, $p<0.0001$). Compared to OA, pairwise Mahalanobis distances between group means were WB<LVB<LVW for metal concentrations and LVB<WB<LVW for biological traits. Respective primary drivers for these separations were Ag, As, Ba, Hg, Pb, Se and Zn; and TL, GSI, 11kt, and Hct. Canonical correlation analysis using the latter variable sets showed they are significantly associated ($p<0.0003$); with As, Ba, Hg, and Zn, and TL, 11kt, and Hct being the primary contributors to the association. In conclusion, male carp collected along a contaminant gradient in LMNRA have distinct, collection site-dependent metal and morpho-physiological profiles that are significantly associated with each other. These associations suggest that fish health and reproductive condition (as measured by the biological variables evaluated in this study) are influenced by levels of certain metals in the Lake Mead environment.

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1. Introduction

Metals and metalloids (hereafter collectively referred to as metals) are ubiquitous in the environment and their source can be difficult to establish as anthropogenic or natural (Dias and Edwards, 2003; Reimann and de Caritat, 2005). However, at local and regional scales, anthropogenic sources of metals in aquatic habitats may include industrial, agricultural and urban runoffs and wastewater discharges (Sorensen, 1991; Short et al., 2008). Lake Mead reservoir was formed by the creation of Hoover Dam along the Colorado River in 1935 and is now part of the Lake Mead National Recreation Area (LMNRA). The reservoir also receives inflow from the Muddy/Virgin Rivers into its Overton Arm (OA), and from Las Vegas Wash (LVW) into Las Vegas Bay (LVB) along the southwest shoreline of Lake Mead. The OA in the original Colorado River basin is upstream from LVB. Las Vegas Wash serves as drainage for municipal wastewater and storm runoff from

Abbreviations: 11kt, 11-ketotestosterone; CANCOR, canonical correlation analysis; CF, Fulton's condition factor; D^2, Mahalanobis distance; DFA, discriminant function analysis; Estradiol-17β, E2; GSI, gonadosomatic index; Hct, hematocrit; LVB, Las Vegas Bay; LMNRA, Lake Mead National Recreation Area; LVW, Las Vegas Wash; WB, Willow Beach.

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the Las Vegas Valley, which includes the City of Las Vegas, Nevada (Reginato and Piechota, 2004). A recent study of sediment cores found generally higher levels of metals and other anthropogenic contaminants in samples taken from LVB compared to other sites in the reservoir, including OA (Rosen and Van Metre, 2010). This study (Rosen and Van Metre, 2010) and others (Bevans et al., 1996; Covay and Leiker, 1998; Goodbred et al., 2007; Leiker et al., 2008; Rosen et al., 2010) have concluded that an important source of anthropogenic contaminants into the reservoir is inflow from LVW.

Teleost fishes accumulate metals in their body by direct uptake from water (via gills) as well as through diet and, although there can be considerable variability in bioaccumulation among species depending on their life history (Short et al., 2008), tissue levels for certain metals generally correspond to those found in their biotic and abiotic environments (Birge et al., 2000; Brumbaugh et al., 2005; Burger et al., 2005; Besser et al., 2007; Luoma et al., 2008; Wang and Rainbow, 2008). Some metals are biologically essential (trace metals) (Watanabe et al., 1997), whereas others are non-essential and can be even toxic at very low levels (Sorensen, 1991). Also, some essential metals, such as Se and Zn, can become toxic at high levels (Sorensen, 1991). Disruptions of the endocrine system in wildlife due to exposure to contaminants, and the consequences of this disruption to natural populations, have received considerable attention in the last two decades (Tyler et al., 1998; Sumpter and Johnson, 2005; Carr and Patiño, 2011). Numerous laboratory and mesocosm studies have addressed the toxicological and endocrinological effects of exposure to metals in teleosts (Sorensen, 1991; Heath, 1995; Kime, 1998). However, studies of endocrine disruption in wild fishes typically do not incorporate an assessment of environmental metals (or metal body burdens) into their analytical designs despite evidence suggesting that bioaccumulation of metals may be associated with biological condition (e.g., Larsson et al., 1985; Haux et al., 1986; Hontela et al., 1995; Couture and Rajotte, 2003; Gravel et al., 2005; Pyle et al., 2005; Hinck et al., 2008), including reproductive condition (Pyle et al., 2005; Hinck et al., 2008).

The goal of this study is to understand relationships between environmental contaminants in LMNRA and the reproductive and endocrine health of its fish populations. Specific objectives are to determine if the elemental composition and morpho-physiological condition of common carp (*Cyprinus carpio*) varies along a previously established gradient of environmental contaminants in the area (Bevans et al., 1996; Covay and Leiker, 1998; Goodbred et al., 2007; Leiker et al., 2008; Rosen and Van Metre, 2010), and the association between these two variable sets. Multivariate statistical approaches were used to address these objectives. Fish used in this study are adult male carp from a larger study whose objective was to examine the relationship between organochlorine contaminant bioaccumulation and biological condition (R. Patiño et al., unpublished data). Specimens for the larger study were collected multiple times from March 2007 through March 2008, but fish used in the present study include only those collected during March 2008. The emphasis of this study was on male carp because an earlier study indicated that males are more sensitive to variation in environmental conditions than females (Patiño et al., 2003). As recommended by a previous study of common carp (Goldstein and DeWeese, 1999), whole-body metal concentrations are reported.

2. Methods

2.1. Fish collection and field processing

Fish collection sites for this study were LVW, which is the sole drainage system into Lake Mead reservoir for wastewater and runoff from the Las Vegas Valley (containing the Cities of Las Vegas and Henderson), Nevada; LVB, which receives urban flows from LVW; OA, an upstream reference site receiving flows from the Muddy/Virgin Rivers that are relatively unimpacted by urban land uses; and Willow Beach (WB), which is on the main stem of the Colorado River approximately 20 river kilometers downstream from Hoover Dam (Fig. 1).

Adult male carp for this study were collected in March (2008) when testicular growth (GSI) is complete or nearly complete prior to the onset of spawning (~April) in Lake Mead (Patiño et al., 2003). Fish were captured with an electrofisher boat near the shoreline in water depths of up to 3 m. They were immobilized by pulsed DC current, captured with dipnets, and maintained in a live well until processed. Fish from each site were collected on consecutive days over a 4-day period in March 2008. Multiple collection trips were conducted in each site, beginning about 0700–0800 h and continuing until the desired number of fish ($n=13$ males) was reached, typically by 1200–1500 h. The time between fish collection and processing was 1–2 h.

Fish were individually processed in the field at a portable laboratory on shore. They were quickly sacrificed by a quick blow to the head and measured for total length and weight. Blood was drawn into heparinized syringes from the caudal vasculature and maintained on wet ice until centrifugation could be conducted later in the day, at which time plasma was separated and stored frozen (−80 °C) for later analysis of the sex steroid hormones, 11-ketotestosterone (11kt) and estradiol-17β (E2). Hematocrit (Hct) was determined on-site at the time of blood sample collection by centrifugation in capillary tubes (HemataSTAT II Microhematocrit centrifuge; Separation Technology, Inc., Altamonte Spring, Florida). The gonads were removed and weighed. Gonadosomatic indices (GSI) and Fulton's condition factor (CF) were determined according to the formulas, (gonad weight/body weight) × 100 and (body weight)/(total length)^3, respectively. Fish carcasses (without the gonads) were wrapped in aluminum foil, placed in a plastic bag and maintained on wet ice until they could be stored frozen (−80 °C) later in the day.

2.2. Metal analyses

Available resources allowed metal analysis in 10 fish from the total of 13 collected per site. The first ten fish collected at each site were chosen for this analysis. Individual fish samples were ground whole from a frozen state to a homogeneous wet mass by LET Inc. (Columbia, MO), and returned to the USGS Columbia Environmental Research Center, where samples were lyophilized to remove moisture. Dried sample material was placed in a plastic bag and further homogenized by crushing with a Teflon roller to achieve a coarse powder consistency. To prepare digestates of fish tissue suitable for semi-quantitative scan by inductively coupled plasma-mass spectrometry (ICP-MS), an aliquant of each dried sample (~0.25 g) was heated with 6 mL nitric acid in a sealed Teflon vessel in a microwave oven and diluted to 100 mL with deionized water. An additional aliquant (~0.5 g) of each dried sample was subjected to a magnesium nitrate–nitric acid dry ashing procedure followed by hydrochloric acid reduction to prepare solutions for the determination of selenium.

A total of 63 elements were analyzed. To perform a "scan" for these elements (excluding Se and Hg), samples were analyzed by ICP-MS (Perkin-Elmer/Sciex Elan 6000) using the semi-quantitative scan mode (TotalQuant). All samples were diluted 10X by a CETAC ASD-500 autodiluter as part of the analytical sequence. Internal standards were Sc (10 ppb), Rh (10 ppb) and Th (10 ppb), and the external standard consisted of a NIST traceable reference solution (Trace Metals in Drinking Water; High Purity Standards, Charleston, SC) to which 5 elements (Pr, Tb, Tm, Ta, and Au) were added for improved calibration in the rare earth region of the mass spectral range. Mercury was determined with a direct mercury analyzer (Milestone DMA-80 analyzer) equipped with an automated sample carousel. With this method, a dried sample (~50 mg) was combusted in a stream of oxygen. All Hg in the sample was volatilized and trapped by amalgamation on a gold substrate and was thermally desorbed and quantified by atomic absorption spectrophotometry. The determination of Se in dry-ashed samples was accomplished by flow injection hydride

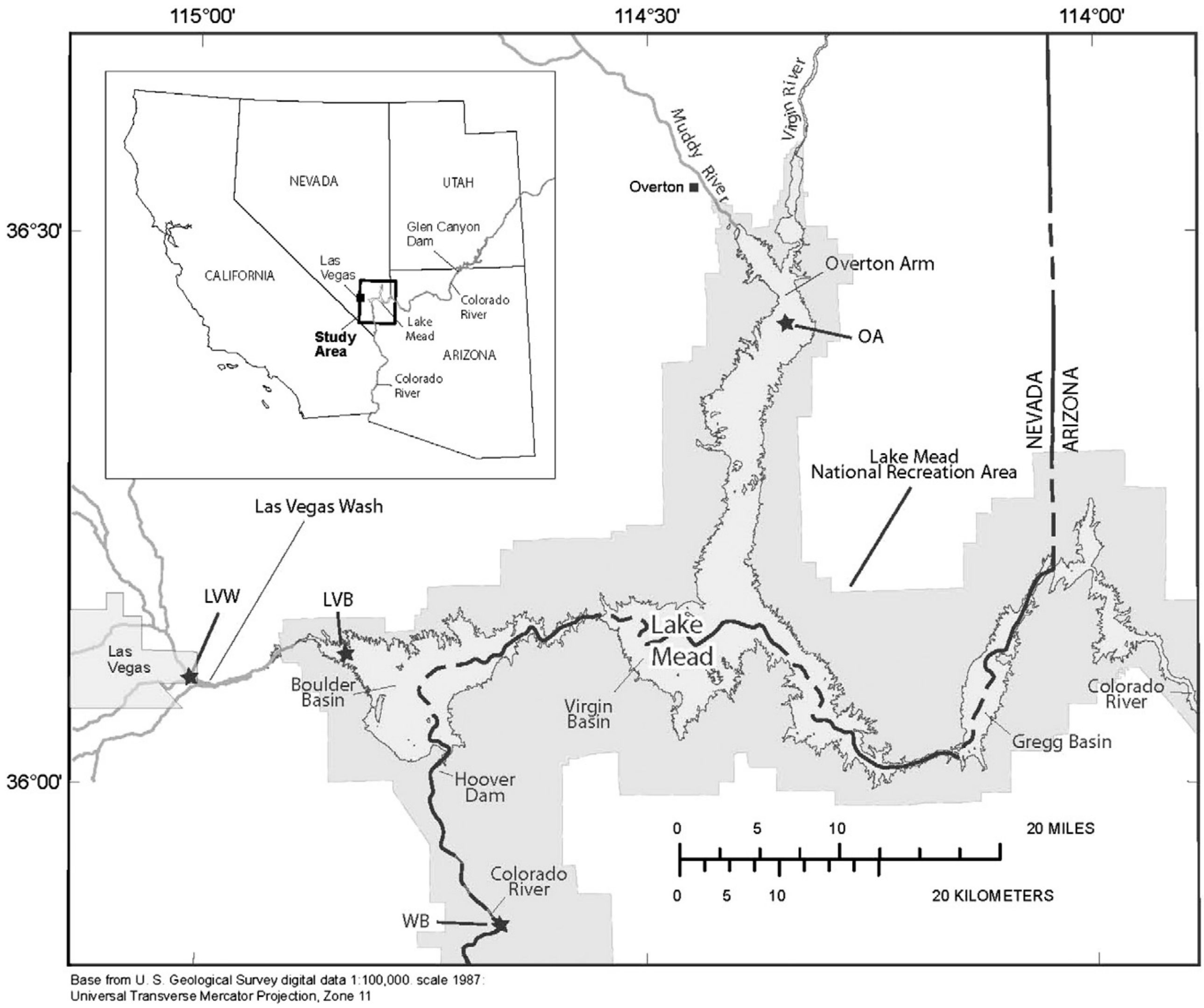

Fig. 1. Map of Lake Mead National Recreation Area. The locations of the sampling sites are shown with asterisks (LVW, Las Vegas Wash; LVB, Las Vegas Bat, WB, Willow Beach; OA, Overton Arm).

generation atomic absorption spectroscopy, where the digestate was mixed with a hydrochloric acid carrier solution and then reduced by sodium tetrahydridoborate which has been stabilized with sodium hydroxide. The resulting volatile hydrogen selenide was transferred with argon carrier gas into a heated quartz cell mounted on an atomic absorption spectrophotometer for decomposition and measurement.

Quality control included method replicates, spikes, and reference materials. For elements measured by semi-quantitative ICP-MS, method precision was determined by triplicate digestion and analysis of two fish samples. Average percent relative standard deviations (%RSDs) were $\leq$25% for elements having two detectable concentrations, with the exception of Br (38%) and Ni (34%). Replicates of fish samples for Se and Hg were $<$10 %RSD. Metals spiked into whole-body fish samples determined by semi-quantitative ICP-MS exhibited recoveries ranging from 72 to 118%. Recoveries of Se and Hg spikes from fish ranged from 73 to 117%. With the exception of Al and Fe, recoveries of elements having certifiable concentrations above detection limits from tissue reference materials (oyster, NIST 1566b; liver, NIST 1577b) ranged from 67 to 136%. Recoveries of Hg and Se from tissue reference materials (fish solubles, IAEA A-6; fish tissue, NIST 1946; dogfish muscle, NRCC DORM-2; dogfish liver, NRCC DOLT-3; copepod, IAEA MA-A-1; tuna muscle, NIST RM50; mussel tissue, IAEA MA-M-2; fish flesh homogenate, IAEA M-A-A-2) ranged from 86 to 126%. Semi-quantitative ICP-MS detection limits (μg/g dry weight) were 0.05 for all elements except Na, Mg, K, and Ca (50); Fe (5); and Ti, Ni,Cu, Zn, Br, Sr, and Ba (0.5). Detection limits for Hg were 0.019 and 0.061 μg/g, and for Se 0.017 μg/g.

2.3. Hormone analyses

Plasma sex steroids were analyzed by enzyme-linked immunosorbent assay (ELISA). The procedures used were those recommended by the kit manufacturer: kit no. 582751 for 11kt and 582251 for E2 (Cayman Chemical Company, Ann Arbor, Michigan, USA). The steroid 11-ketotestosterone was measured directly in plasma but E2 measurement required solvent-extraction to reduce matrix effects. Extraction efficiencies for E2 were determined in individual samples using radiotracer methods and the ELISA results were accordingly corrected. Detection limits were 102 pg/mL for 11kt and 7 pg/mL for E2.

2.4. Statistical analyses

Statistical analyses were conducted using the SAS 9.2 software package (SAS Institute Inc., Cary, North Carolina) with a significance level of $\alpha = 0.05$. Undetectable values were assigned one-half of the detection limits for their respective assays and generally only variables with overall $<$15% non-detection rates were included in the analysis. Metal and 11kt values were log-transformed, and Hct and

GSI were converted to the arcsine of their square root prior to analysis. These data transformations generally improved homogeneity of variances and normality of distributions. The other variables met parametric assumptions and were not transformed. Body weight was not included in the analysis of biological traits because CF already contains this information (corrected for total length), and because there also are limitations to the number of variables that can be included in multivariate analyses relative to the number of samples (McGarigal et al., 2000).

Preliminary assessments of fish condition (metal concentrations and biological traits) at the various collection sites were conducted using one-way or Kruskal–Wallis ANOVAs followed by appropriate multiple comparison (Tukey's or Dunn's), but the principal analytical tool for assessing these differences was discriminant function analysis (DFA; SAS DISCRIM procedure). The purpose of this multivariate procedure is to describe maximum differences among pre-specified sample groups by reducing the multidimensionality of datasets to a smaller number of new composite dimensions (canonical functions) (McGarigal et al., 2000). Differences between group (canonical) means or centroids are expressed by means of pairwise Mahalanobis distances (D^2), and the strength of the relationship between each original variable and their respective canonical function is indicated by structure coefficients (McGarigal et al., 2000). Classification matrices were generated for the purpose of assessing the stability of canonical functions. Because of the relatively limited sample size, validation of canonical functions for metal analysis was conducted by comparing the classification of specimens based on the original functions to the classification obtained following resampling procedures (Jackknife validation; McGarigal et al., 2000). For analysis of biological traits, the availability of additional fish (total $n = 12$; 3/site) that were not included in the original derivation of canonical functions allowed a split-sample approach for testing function stability (McGarigal et al., 2000).

Canonical correlation analysis (CANCOR; SAS CANCORR procedure) is related to DFA in that it also reduces the dimensionality of multivariate data sets into a smaller number of larger groupings (canonical variates) with minimum loss of information, thus simplifying an understanding of overall relationships (McGarigal et al., 2000). Although a distinction between variable sets is not mathematically necessary, for this study we considered whole-body metal concentrations as the independent variable set and biological traits as the dependent variable set. In this context, the measure of "redundancy" or shared variance is the ability of canonical variates for the independent set (e.g., metal concentrations) to explain or account for the variation in the dependent set (e.g., biological traits) (McGarigal et al., 2000). Like DFA, structure coefficients are used in CANCOR to assess the strength of the association between individual variables in either set and their corresponding canonical variates.

Pearson's correlation coefficients for selected metal-biology pairwise associations are also reported in this study to help in the interpretation of canonical results. The primary purpose of this bivariate analysis is only to characterize the relative strength of the selected pairwise associations. Thus, their *p*-values are not reported as they would be misleading without proper α correction for the multiple analyses.

3. Results

3.1. Metals

Whole-body concentrations for the majority of the 63 elements analyzed were below, or mostly below, detection limits. Twenty-five elements had an overall non-detection rate of <15% and are reported in this study (Table 1). Values for Cd are also reported despite a high non-detection rate (overall 49%) because cursory examination of these data suggested differences among sites; i.e., non-detectable values seemed to be associated with fish from specific sites, such as LVW where all values were below the detection limit (Table 1). Appropriate one-way ANOVA and multiple comparison tests (parametric or non-parametric) were conducted on each element; the purpose of this univariate analysis was to gain insights of trends in metal composition and, in a few cases, to aid in the selection of metals for multivariate analysis. Generally, fish from LVW seemed to have the highest levels of Ag, As, Mo, Ru, and Zn; and lowest levels of Ba, Cd, Co, Fe, Hg, Pb, U, and V (Table 1). Fish from WB had the lowest levels of Li, Mg and Sr; fish from OA had the highest levels of Se; and fish from LVB did not seem to clearly place as uniquely highest or lowest in their concentration for any of the metals reported in this study (Table 1).

Fourteen of the 26 elements reported (Table 1) are considered in the United States as being of potential concern to the quality of freshwater habitats: Ag, Al, As, Ba, Cd, Co, Cr, Cu, Fe, Hg, Mn, Pb, Se, Zn (USEPA, 2009). Four of these elements, Al, Cr, Cu, and Mn seemed to have similar concentrations among sites (Table 1). Thus, to reduce possibility of complications due to low sample size-to-variable ratios (McGarigal et al., 2000), metals used in the multivariate analysis included only the remaining 10 elements (Table 1). Discriminant function analysis yielded a significant model (Wilk's $\lambda = 0.03$; $F(30,79) = 6.32$; $p < 0.0001$) with three significant canonical functions bearing eigenvalues of 4.05, 2.95 and 0.78, respectively. Metals with structure coefficients ≥ 0.3 were Ag (−0.33), As (−0.39), Ba (0.48), Hg (0.43), and Pb (0.45) on the first function; Ag (−0.31), Ba (0.30), and Se (0.44) on the second; and Se (0.39) and Zn (0.42) on the third (structure coefficients are shown in parentheses).

A biplot of data on the first and second canonical functions showed that fish are organized into distinct groups corresponding to their respective collection sites; however, some overlap exists between OA and WB (Fig. 2), an observation which is also evident in the small D^2 between these two groups (Table 2). Arsenic, Ba and Hg and Pb seemed to be the primary metals separating LVW fish from the others along function 1; whereas OA, WB and LVW seemed to be separated among themselves primarily by Ag and Se, and to a lesser degree Ba, along function 2 (Fig. 2).

The overall classification of samples based on their metal composition was 90% correct when the original samples were used, and 72.5% following resampling (Table 3). The lowest performance was for OA samples, which were misclassified as belonging to WB 30% (original) and 40% (resampling) of the time. This observation is also consistent the low D^2 between OA and WB (Table 2) as well as the graphical overlap in the biplot (Fig. 2); however, the classification rate for WB samples was better than for OA samples (Table 3). Although the reduced classification rate after resampling indicates a degree of instability of the functions – perhaps due to a suboptimal sample-to-variable ratio – the overall posterior classification rate of 90% is adequate for the descriptive purposes of the present study.

3.2. Biological traits

Estradiol-17β values were above detection limits in all samples, and 11kt was undetectable in only one sample (of 13) from LVW. Generally, mean TL was similar between LVB and WB and higher at these sites than at OA and LVW, where TL was also similar; mean GSI was largest at OA and declined, in order, at LVB, LVW and WB (with WB being nearly half of the value at OA); Hct was lowest at OA and WB and highest at LVW; and 11kt was highest at OA and LVB and lowest at LVW (Table 4). Condition factor and E2 were similar among all groups (Table 4).

All biological variables were included in the DFA. This analysis yielded a significant model (Wilk's $\lambda = 0.04$; $F(18,88) = 10.50$; $p < 0.0001$) with three significant canonical functions bearing eigenvalues of 5.73, 1.74 and 0.38, respectively. Traits with structure coefficients ≥ 0.3 were 11kt (−0.66) on the first function; TL (−0.82) and GSI (0.41) on the second; and TL (0.44), GSI (0.71) and Hct (0.38) on the third (structure coefficients are shown in parentheses).

Table 1
Metal and metalloid concentrations in whole body of male common carp (μg/g dry weight) collected from various sites in Lake Mead National Recreation Area (OA, Overton Arm; LVB, Las Vegas Bay; LVW, Las Vegas Wash; WB, Willow Beach). Values shown are the means (SEM) of measurements in 10 fish per site. Percent tissue moisture levels (moist.) are also shown. For each variable, values associated with common letters are not significantly different among sites ($p<0.05$). Metals that met the selection criteria for inclusion in discriminant function analysis are shown in italics.

	OA	LVB	LVW	WB		OA	LVB	LVW	WB		OA	LVB	LVW	WB		OA	LVB	LVW	WB
Ag	0.088^{A}	0.119^{AB}	0.160^{B}	0.097^{A}	Cr*	2.80^{A}	1.73^{A}	2.40^{A}	1.90^{A}	Mg*	1297^{A}	1129^{AB}	1285^{A}	921^{B}	Sr*	269^{A}	235^{AB}	275^{A}	141^{B}
	(0.004)	(0.014)	(0.016)	(0.002)		(0.44)	(0.24)	(0.40)	(0.18)		(127)	(71)	(52)	(65)		(40)	(39)	(35)	(17)
Al	444.1^{A}	69.4^{A}	69.3^{A}	49.8^{A}	*Cu*	5.10^{A}	4.70^{A}	5.90^{A}	4.90^{A}	Mn	19.5^{A}	12.1^{A}	16.4^{A}	11.3^{A}	Ti*	53.5^{A}	42.5^{A}	46.5^{A}	35.8^{A}
	(244.5)	(27.7)	(24.0)	(11.2)		(0.46)	(0.37)	(0.18)	(0.31)		(6.3)	(2.3)	(1.7)	(1.3)		(6.9)	(4.1)	(2.8)	(3.1)
As	0.83^{AB}	0.76^{A}	1.55^{B}	0.81^{AB}	*Fe*	754^{A}	564^{A}	405^{B}	492^{AB}	Mo	0.075^{A}	0.094^{A}	0.158^{B}	0.094^{AB}	U	0.214^{A}	0.216^{A}	0.067^{B}	0.200^{A}
	(0.06)	(0.16)	(0.19)	(0.07)		(162)	(27)	(37)	(25)		(0.008)	(0.035)	(0.027)	(0.014)		(0.055)	(0.054)	(0.009)	(0.026)
*Ba**	16.2^{A}	10.3^{A}	3.6^{B}	10.4^{A}	Ga	0.440^{A}	0.260^{AB}	0.210^{B}	0.230^{AB}	Na*	4800^{A}	4430^{A}	4490^{A}	4120^{A}	V	1.29^{A}	0.83^{AB}	0.41^{B}	0.80^{AB}
	(3.0)	(1.4)	(0.3)	(1.4)		(0.098)	(0.031)	(0.028)	(0.021)		(457)	(361)	(214)	(282)		(0.39)	(0.16)	(0.09)	(0.05)
Ca*	46610^{A}	41150^{A}	42420^{A}	33350^{A}	*Hg*	0.175^{A}	0.183^{A}	0.034^{B}	0.129^{A}	*Pb*	0.60^{A}	1.10^{A}	0.20^{B}	0.45^{AB}	*Zn*	224^{A}	279^{A}	332^{B}	182^{A}
	(5187)	(5126)	(3448)	(4132)		(0.023)	(0.040)	(0.006)	(0.013)		(0.11)	(0.21)	(0.03)	(0.05)		(20)	(69)	(19)	(15)
Cd	0.144^{A}	0.151^{AB}	0.025^{B}	0.116^{A}	K	12750^{A}	12410^{A}	13360^{A}	11340^{A}	Ru	4.60^{A}	3.50^{A}	6.70^{B}	3.80^{A}	Moist.	72.0 ^{A}	71.2 ^{A}	72.7 ^{A}	70.4 ^{A}
	(0.038)	(0.064)	(0)	(0.046)		(730)	(440)	(394)	(479)		(0.56)	(0.27)	(0.45)	(0.20)		(0.8)	(1.1)	(0.7)	(1.2)
Co	0.266^{AB}	0.164^{AB}	0.114^{B}	0.230^{A}	Li	0.840^{A}	0.550^{AB}	0.370^{AB}	0.260^{B}	*Se**	8.12^{A}	5.10^{B}	4.71^{B}	5.51^{B}					
	(0.063)	(0.032)	(0.019)	(0.026)		(0.292)	(0.182)	(0.042)	(0.031)		(0.40)	(0.55)	(0.51)	(0.36)					

*1-way ANOVA and Tukey's multiple comparison tests on log-transformed values; all other metals, Kruskal–Wallis ANOVA and Dunn's multiple range test; %moisture levels, 1-way ANOVA and Tukey's multiple comparison tests on Arcsin(SqRt)-transformed values.

A biplot of the data between the first and second functions showed that fish are organized into groups corresponding to their respective collection sites; however, considerable overlap exists between LVB and WB (Fig. 3), an observation which is also evident in the relatively small D^2 between these two groups (Table 2). 11-ketotestosterone seemed to be the primary biological variable separating LVW fish from all others along function 1, although TL also seemed to separate LVW from LVB and WB but not from OA; whereas the remaining three groups (OA, LVB, WB) seemed to be separated among themselves primarily by TL and GSI and to some degree also by 11kt, along function 2 (Fig. 3). (Note: the contribution of Hct to group separation cannot be shown on Fig. 3 because the Hct contribution is associated with the third function.)

The overall classification of samples based on their biological traits was 87.5% correct when the original samples were used, and 91.7%

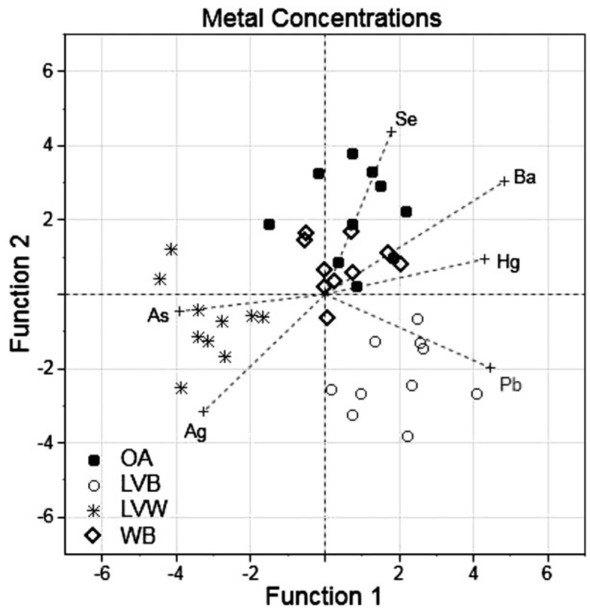

Fig. 2. Discriminant function plot of metal concentration data for common carp collected from four different sites in Lake Mead National Recreation Area (Overton Arm, OA; Las Vegas Bay, LVB; Las Vegas Wash, LVW; Willow Beach, WB). Vectors representing the standardized coefficients for each metal are superimposed on the plot to indicate each metal's contribution to the discrimination between sites (vector lengths are relative to each other and not according to the axes scales). Only those metals with coefficients ≥ 0.80 on the first two discriminant functions are shown on the plot.

following (Jackknife) classification of the extra samples (Table 3). These observations confirm the stability of the discriminant model for biological traits.

3.3. Relationship between metal concentrations and biological traits

Only metal or biological variables with factor structure coefficients ≥ 0.3 in their respective DFAs were used in CANCOR in order to limit the number of variables and reduce complications due to low sample-to-variable ratio. The selected variables thus were Ag, As, Ba, Hg, Pb, Se, Zn for metals and TL, GSI, Hct, 11kt for biological traits (see Sections 3.1 and 3.2). A substantial and highly significant correlation between the two canonical variable sets was observed (canonical $R=0.82$; Wilk's $\lambda=0.16$; $F(28,105.98)=2.54$; $p<0.0003$), with only the first variate pair contributing to the significance of the association. The pattern of data scatter on the correlation plot indicated a reasonable alignment of samples from OA, WB and LVW in regards to the regression line – with LVW samples once again occupying a clearly distinct position on the plot. However, samples from LVB seemed to align in parallel to the x-axis (Fig. 4), perhaps suggesting minimal or no association between metal composition and biological variables in fish from this site.

The fraction of standardized variance (redundancy) in biological traits that is explained by the first canonical variate of metal concentrations was 0.23. Because this redundancy value is relatively low, only variables bearing CANCOR structure coefficients ≥ 0.6 with respect to their corresponding canonical variates were interpreted in this study. Thus, primary variables contributing the canonical correlation include Ba (−0.75), As (0.66), Hg (−0.63) and Zn (0.62) for metal concentrations; and Hct (0.74), TL (−0.66) and 11kt (−0.61) for biological traits (structure coefficients are shown in parentheses). Simple bivariate correlations between metal concentrations and biological traits are generally consistent with

Table 2
Squared Mahalanobis distances between group (site) means for metal concentration and biological trait data of male common carp collected from four sites in Lake Mead National Recreation Area (OA, Overton Arm; LVB, Las Vegas Bay; LVW, Las Vegas Wash; WB, Willow Beach).

Metal concentrations				Biological traits			
Site	OA	LVB	LVW	Site	OA	LVB	LVW
LVB	20.7*			LVB	11.1*		
LVW	24.1*	28.3*		LVW	40.6*	26.3*	
WB	7.1	13.9*	17.9*	WB	16.2*	3.6	26.9*

*Overall $p<0.05$.

Table 3
Classification matrix of male common carp collected from four sites in Lake Mead National Recreation Area (OA, Overton Arm; LVB, Las Vegas Bay; LVW, Las Vegas Wash; WB, Willow Beach). Predicted classifications were based on discriminant function analysis of metal concentration and biological trait data.

Metal concentrations		Percent correct	Predicted classification: OA ($p=.25000$)	LVB ($p=.25000$)	LVW ($p=.25000$)	WB ($p=.25000$)
Original cases[a]	OA	70	7	0	0	3
	LVB	100	0	10	0	0
	LVW	100	0	0	10	0
	WB	90	1	0	0	9
	Overall	90	8	10	10	12
Resampling[b]	OA	50	5	0	1	4
	LVB	70	1	7	2	0
	LVW	90	0	0	9	1
	WB	80	1	1	0	8
	Overall	72.5	7	8	12	13

Biological traits		Percent correct	Predicted classification: OA ($p=.25000$)	LVB ($p=.25000$)	LVW ($p=.25000$)	WB ($p=.25000$)
Original cases[a]	OA	90	9	1	0	0
	LVB	80	1	8	0	1
	LVW	100	0	0	10	0
	WB	80	0	2	0	8
	Overall	87.5	10	11	10	9
New cases[c]	OA	100	3	0	0	0
	LVB	100	0	3	0	0
	LVW	67	1	0	2	0
	WB	100	0	0	0	3
	Overall	92	4	3	2	3

[a] Posterior classification based on the original cases ($n=10$ per site; total $n=40$).
[b] Reclassification based on Jackknife resampling.
[c] A priori classification based on new cases ($n=3$ per site; total $n=12$).

results of CANCOR, especially with respect to the relative lack of substantial associations between GSI and any of the individual metals used in the analysis (Table 5).

4. Discussion

Male common carp from LMNRA could be statistically separated by collection site based on either their metal composition or morpho-physiological condition. For both sets of variables, LVW fish were the most distinctly unique when contrasted with fish from all other sites. Also, when OA is used as reference, the relative magnitude of pairwise group discrimination was, from low to high, WB<LVB<LVW for metal composition and LVB<WB<LVW for biological variables. Thus, the metal composition and biological condition of male common carp in LMNRA is at least partly associated with the known gradient of environmental contamination; namely, (1) the OA region of Lake Mead reservoir is the upstream (reference) site for this study; (2) LVW carries urban runoff and wastewater effluent from the Las Vegas Valley metropolitan area – with a relatively high contaminant load – into LVB, which serves as the mixing zone between LVW and the rest of Lake Mead (Bevans et al., 1996; Covay and Leiker, 1998; Reginato and Piechota, 2004; Goodbred et al., 2007; Rosen and Van Metre, 2010; Rosen et al., 2010); and (3) WB is downstream of the dam.

Among the metals analyzed in this study, those that contributed the most to the strength of the discriminant functions were Ag, As, Ba, Hg, Pb, Se and Zn. With the exception of Se and Zn, these metals are non-essential. Inspection of the biplot (Fig. 2) and the descriptive statistics for each metal (Table 1) indicated that LVW fish separated from the other groups primarily on the basis of As (higher body burdens than all other groups), Ba (lower), Hg (lower), Pb (lower) and Zn (higher). Las Vegas Wash and LVB fish had similar levels of Ag, which were higher than at the other two sites; and OA had higher levels of Se. These observations suggest that not all metal body burdens follow the contaminant gradient in LMNRA as defined by the premise that LVW is the primary source of anthropogenic contaminants. In fact, LVW fish had the lowest concentrations observed for Ba, Hg and Pb; and OA fish, the reference group for this study, had the highest concentrations of Se. A recent study of metals in Lake Mead sediment cores (Rosen and Van Metre, 2010) provides useful insight for the interpretation of these findings. Namely, the study found that As and Zn were higher in LVB sediment than other regions of the reservoir, including OA (LVW and WB were not sampled), and concluded that the source of these two metals was flow from LVW (Rosen and Van Metre, 2010); a conclusion which is consistent with the present finding that As and Zn levels were highest in fish form LVW. The same study found higher sediment levels of Hg in regions of the reservoir outside of LVB (OA was not analyzed for Hg), and suggested that a major determinant for the relative distribution of Hg in Lake Mead reservoir sediment is the historical pattern of upstream flow in the Colorado River watershed (Rosen and Van Metre, 2010); once again, a suggestion consistent with the present finding that Hg was lowest in fish from LVW. The only metal for which there may be disagreement between reservoir sediment and fish body burdens is Pb, which showed higher concentrations in sediment from LVB (presumably of LVW origin) than other sites of the reservoir (Rosen and Van Metre, 2010), but lower body burdens in fish from LVW (present study). Barium, Ag and Se were not examined in the sediment study (Rosen and Van Metre, 2010); however, a primary source of Se in the lower Colorado River Basin (which includes LMNRA) is believed to be the upper Colorado River Basin (Hinck et al., 2007). Irrigation associated with agriculture and related rural land uses within the OA watershed are also likely to contribute loading of Se and other trace elements to LMNRA. Overall, with some possible exceptions (e.g., Pb), metal body burdens of male common carp from LMNRA appear to reflect the general metal composition of their immediate habitat. These observations and conclusions are consistent with those of earlier studies with teleosts suggesting that, for a given species, body burdens for a number of metals vary among populations in general accordance to the metal composition of their respective biotic and abiotic environments (Hontela et al., 1995; Saiki et al., 1995; Birge et al., 2000; Laflamme et al., 2000; Brumbaugh et al., 2005; Burger et al., 2005; Luoma et al., 2008; Wang and Rainbow, 2008). However, not all potential sources of metals to common carp in Lake Mead have been accounted for in this study as there are limited or no data on metals in water or carp diet at the various collection sites. In addition, because body concentrations of some metals may be regulated to some extent (Sorensen, 1991), associations between environmental and body metal concentrations have to be interpreted with caution.

Gonadal development in adult common carp of Lake Mead is fairly synchronous during a period of several months leading to the onset of the spawning season (Patiño et al., 2003). Fish for this study were sampled an estimated few weeks before spawning to minimize individual variability and also to allow data interpretation in the context of spawning condition. Plasma 11-ketotestosterone, Hct, GSI and TL were the primary biological variables contributing to fish separation by collection site. Fish from LVW separated from all others based on 11kt and Hct (lower and higher, respectively, at LVW); and fish from OA separated from LVB and WB on the basis of GSI (highest at OA) and TL (lowest at OA) (Fig. 3 and Table 4). Condition factor and plasma levels of E2 did not contribute to the separation. These observations are generally

Table 4
Total length (TL, mm), Fulton's condition factor (CF), gonadosomatic index (GSI, %), hematocrit (Hct, %) and plasma 11-ketotestosterone (11kt, pg/ml) and estradiol-17β (E2, pg/ml) levels in male common carp collected from four sites in Lake Mead National Recreation Area (OA, Overton Arm; LVB, Las Vegas Bay; LVW, Las Vegas Wash; WB, Willow Beach). Values shown are the means (SEM) of measurements in 10 fish per site. For each variable, means associated with common letters are not significantly different among sites ($p<0.05$). Biological traits that met the selection criteria for inclusion in discriminant function analysis are shown in italics.

	OA	LVB	LVW	WB		OA	LVB	LVW	WB
*TL**	450^{A} (7)	521^{B} (14)	415^{A} (10)	503^{B} (11)	*Hct*	32.9^{A} (2)	37.1^{AB} (1)	41.8^{B} (1)	34.5^{A} (1)
CF*	1.29^{A} (0.03)	1.33^{A} (0.04)	1.40^{A} (0.02)	1.39^{A} (0.05)	*11kt*	22249^{A} (3994)	12207^{A} (4011)	558^{B} (137)	3467^{AB} (378)
*GSI**	6.9^{A} (0.4)	5.9^{A} (0.5)	5.3^{AB} (0.5)	3.9^{B} (0.6)	E2*	247^{A} (31)	211^{A} (19)	174^{A} (16)	191^{A} (23)

*1-way ANOVA and Tukey's multiple comparison tests (GSI values were transformed to arcsine of square-root); others, Kruskal–Wallis ANOVA and Dunn's multiple range test.

consistent with those of an earlier Lake Mead study reporting that male common carp from LVB were of larger size and yet had lower GSI than fish from OA (Patiño et al., 2003). As previously noted (Patiño et al., 2003), the larger size of fish from LVB may be explained by the higher primary productivity in this region of the reservoir; whereas the relatively lower GSI at LVB (present study; Patiño et al., 2003), LVW and WB (present study) compared to OA could indicate environmental impacts on the testicular growth of fish at these sites. The androgen, 11kt, is essential for fish spermatogenesis (Schulz et al., 2010). Thus, the present observation of lower 11kt in male carp from LVW just before the onset of the spawning period may indicate a degree of spermatogenic impairment in these fish, which in turn could also affect reproductive fitness. Although contaminant-induced changes in Hct levels can potentially impact the oxygen carrying capacity of blood (Larsson et al., 1985), the biological relevance (or consequence) of the relatively high Hct values in male carp from LVW (average, 41.8%) is uncertain. Except for OA (average, 32.9%), Hct values in fish from all other sites (range of

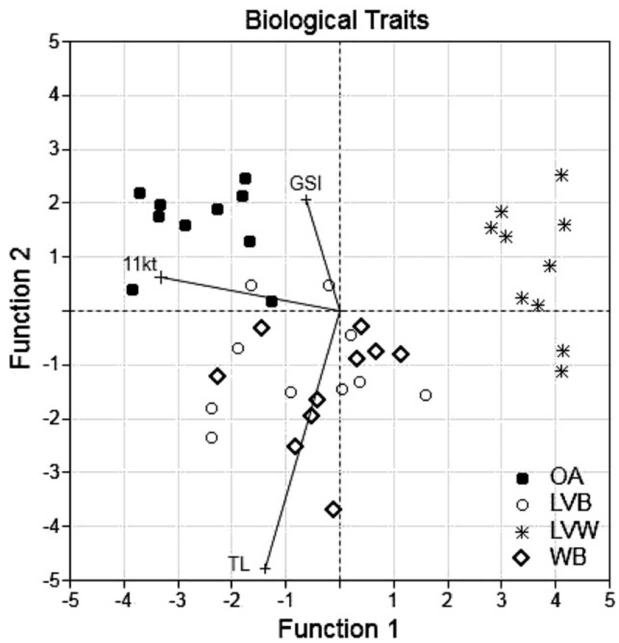

Fig. 3. Discriminant function plot of biological traits for common carp collected from four different sites in Lake Mead National Recreation Area (Overton Arm, OA; Las Vegas Bay, LVB; Las Vegas Wash, LVW; Willow Beach, WB). Vectors representing the standardized coefficients for each biological trait are superimposed on the plot to indicate each trait's contribution to the discrimination between sites (vector lengths are relative to each other and not according to the axes scales). Only those traits with coefficients ≥ 0.80 on the first two discriminant functions are shown on the plot.

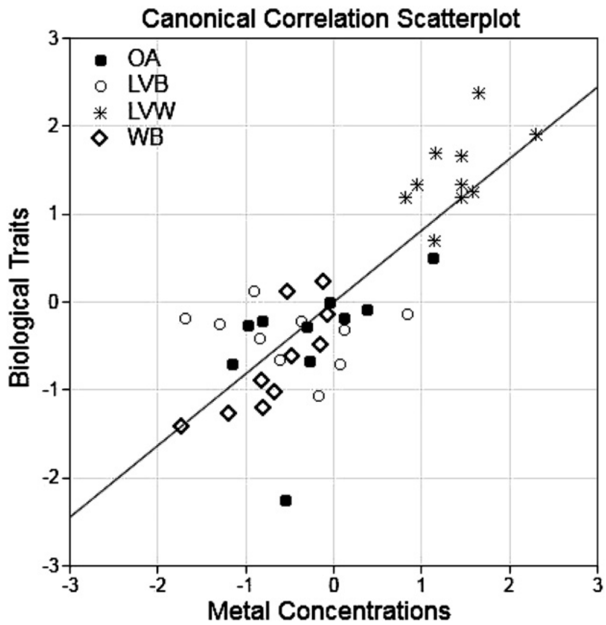

Fig. 4. Scatterplot of the first canonical variate pair derived from canonical correlation analysis of metal concentrations and biological traits in common carp from Lake Mead National Recreation Area. The regression line is shown with the plot.

averages, 34.5–41.8%) were above the typical range (29.7–33.8%) for adult common carp (Hrubec and Smith, 2010). Overall, based on current understanding of the biological variables examined in this study, male carp biological condition seemed to gradually decrease, in order, from OA to LVB, WB and LVW. It is noteworthy, however, that on the basis of GSI alone, WB fish were the farthest from OA fish. Because GSI is an important index of reproductive condition, the interpretation of fish separation by biological condition along a linear gradient relative to OA – namely, placing LVW fish behind WB fish – should be observed with caution. In fact, a full seasonal study of male carp from LMNRA revealed the occurrence of severe testicular abnormalities in some male fish from WB (R. Patiño et al., unpublished data).

Canonical correlation analysis yielded a statistically highly-significant association between metal concentrations (Ag, As, Ba, Hg, Pb, Se, Zn) and biological traits (TL, GSI, Hct, 11kt). Although only 23% of the variance in biological traits was accounted for by the variance in metal concentrations, this fraction of shared variance is substantial considering the numerous physical and chemical factors that affect reproduction and its timing in fishes (Patiño, 1997). Some of the specific biological variables examined in this study (e.g., 11kt, GSI) also experience large seasonal variations (Patiño et al., 2003; R. Patiño et al., unpublished data) that are driven by natural environmental factors, such as photoperiod and temperature. [Fish for this study were collected when testicular growth is complete or nearly complete just prior to the onset of breeding (Patiño et al., 2003)]. Interestingly, the association between metal and biological variable sets was weak or non-existent for fish within LVB. This observation suggests that the biological condition of male carp from LVB is unrelated to metal body burdens within the concentration ranges observed in fish from this site. At the landscape level, the association between whole-body metal content and biological condition was largely driven by the clear separation of LVW fish from the other groups along the regression line.

Closer inspection of CANCOR results indicated that the association between the two variable sets was based primarily on As, Ba, Hg, and Zn; and TL, 11kt and Hct. Canonical correlation analysis is not designed for evaluating pairwise associations between individual metal and biological variables. In fact, the relevance of inspecting simple bivariate associations could be questioned because, under most circumstances, they may rarely occur in natural ecosystems where organisms are exposed to complex

Table 5
Pearson's correlation coefficients (*r*) between metal and biological variables used in canonical correlation analysis for male common carp collected from various sites in Lake Mead National Recreation Area. Fish from all sites were pooled for this analysis (*n* = 40). See text for data transformations.

	TL	11kt	GSI	Hct	Ag	As	Ba	Hg	Pb	Se
11kt	0.40									
GSI	−0.08	0.42								
Hct	−0.08	−0.27	0.04							
Ag	−0.22	−0.39	−0.11	0.44						
As	−0.37	−0.44	−0.08	0.39	0.48					
Ba	0.24	0.51	−0.05	−0.51	−0.53	−0.37				
Hg	0.25	0.39	0.14	−0.50	−0.54	−0.47	0.59			
Pb	0.36	0.24	−0.03	−0.19	−0.35	−0.15	0.59	0.45		
Se	−0.18	0.41	0.29	−0.19	−0.47	−0.48	0.42	0.26	0.09	
Zn	−0.33	−0.35	0.05	0.36	0.18	0.53	−0.25	−0.19	0.05	−0.25

mixtures of natural and anthropogenic chemicals. However, bivariate correlations can be useful to develop and assess hypotheses concerning cause-effect relationships. Thus, the framework used in this study for interpreting bivariate data is: which of the biological (dependent) variables is most strongly associated with the concentration of a given metal (independent variable) in the model?

Whole-body As concentration was relatively-strongly and negatively associated with 11kt, with fish from LVW having the highest levels of As (average, 1.55 μg/g dry weight) and lowest levels of 11kt (average, 558 pg/ml). These observations suggest a cause–effect relationship between increased body-burdens of As and reduced 11kt production. In fact, experimental evidence for such relationship was recently reported for Japanese eel (*Anguilla japonica*), where waterborne exposures of testicular fragments to As at concentrations as low as 7 ng/mL effectively inhibited 11kt production (Celino et al., 2009).

Whole-body Zn was also relatively-strongly and negatively associated with 11kt, with fish from LVW also having the highest levels of Zn (average, 332 μg/g dry weight). These observations suggest that elevated body burdens of Zn may also have contributed to decreased production of 11kt in LVW fish. Whereas at trace levels Zn is an essential element with many physiological activities in teleosts (Watanabe et al., 1997), including a positive influence on spermatogenesis (Yamaguchi et al., 2009), at elevated levels Zn can also function as a remarkably strong reproductive toxin (Brungs, 1969). It should be noted that Zn levels can be naturally high in common carp. In a nationwide survey, the average whole-body Zn content of male carp was 63.2 μg/g wet weight (Hinck et al., 2008); in the present study, the moisture-corrected average value for male carp from LVW was ~89.9 μg/g wet weight, or about 30% higher than the national average. Overall, the negative associations between As/Zn and 11kt in male common carp of this study are consistent with previously reported negative effects of these metals on androgen synthesis and reproduction.

Whole-body Ba was relatively-strongly associated with 11kt but, unlike As and Zn, the relationship was positive (Ba levels were lowest at LVW). There is little information about the toxicological effects of Ba. In a study with leopard coral grouper (*Plectropomus leopardus*), acute exposure to Ba by intraperitoneal injection of 2 or 4 mg/kg body mass (concentration roughly equivalent to the wet-weight content of Ba in LVW fish) had no effect on any of the morpho-physiological variables examined in the fish, including circulating levels of 11kt, E2, testosterone and cortisol (Williamson et al., 2009). Therefore, the present finding of a positive association between whole-body Ba levels and 11kt in male carp is difficult to interpret unless the assumption is made that it is a decreasing level of Ba that drives the association. However, to our knowledge, Ba has no known physiological functions.

Whole-body Zn levels showed a positive correlation and Ba levels a negative association with Hct; and for both metals the strength of the association with Hct was similar or equal in absolute value to their association with 11kt. Also, the strongest association for whole-body Hg was with Hct, and this association was negative. The relevance of these metal-Hct associations is uncertain. Previous studies of the relationship between metal exposure and Hct in teleost have yielded inconsistent results, sometimes showing positive and negative associations for the same metal; e.g., for Zn (Folmar, 1993; Kori-Siakpere and Ubogu, 2008) and Hg (Shah and Altindag, 2004; Oliveira Ribeiro et al., 2006). In the only study of hematological effects of Ba in teleosts available to date, acute exposure of leopard coral grouper to Ba had no effect on Hct for up to 8 weeks after dosing (Williamson et al., 2009). Overall, the information currently available is insufficient to evaluate the relevance of the associations between tissue metals (Zn, Hg, Ba) and Hct observed in this study.

A previous study of yellow perch (*Perca flavescens*) sampled along a metal contaminant gradient suggested that male GSI positively associates (bivariate analysis) with liver and muscle content of Se (Pyle et al., 2005). In the present study with male common carp, GSI contributed substantially to the discrimination of fish by site, and OA fish coincidentally had the highest mean GSI as well as whole-body Se content. However, results of correlation analyses (canonical and bivariate) showed that GSI is the only biological variable that did not contribute substantially to the association with whole-body metal contents, including Se. The apparent differences between the studies with perch (Pyle et al., 2005) and carp (present study) are difficult to interpret because of the different tissue compartments used to determine metal content; the apparent differences in data handling for correlation analysis [individual fish in present study and, apparently, site averages in Pyle et al. (2005; see Tables 13 and 14)]; and possible differences in physiological responses to metal exposure between species. It is also notable that although GSI and 11kt were relatively-strongly associated with each other in the present study, only 11kt substantially associated with tissue metal composition. This later observation indicates that GSI in male carp is also responding to or associated with other environmental or biological conditions not examined in the present study.

Several studies have reported associations between tissue metal concentrations and fish size (length or weight). In general, for metals such as Hg the association has been positive but for other metals, results have been inconsistent (e.g., Barak and Mason, 1990; Goldstein and DeWeese, 1999; Canli and Atli, 2003). In the present study, none of the metals reported from the CANCOR analysis as being associated with biological condition (As, Ba, Hg, and Zn) showed relatively-strong associations with body length; namely, in all cases, the association of the metal was stronger for a biological condition other than total length. This observation is consistent with the results of a previous study of metal concentrations in common carp, which also found no significant associations between fish size and whole-body concentrations for a number of metals (Goldstein and DeWeese, 1999).

A recent nationwide fish survey (Hinck et al., 2008) yielded certain results and interpretations for male common carp that differed from those of the present study. For example, unlike the result of the present study, neither As or Zn were associated with 11kt; and Hg, Mo and Cd showed significant associations with GSI (Hinck et al., 2008). However, the results of these two studies have to be compared with caution because (1) whereas the nationwide survey sampled fish in summer and fall when testes are regressed or in the early process of recrudescence, the present study used spawning-ready males collected in early spring; and (2) whereas the nationwide survey used multiple regression, which examines one dependent (biological) variable at a time, the present study used CANCOR as primary analysis of relationships between full sets of variables. Perhaps the most important of these differences between the studies is date of sampling. Fish reproduction is a cyclic event and levels of reproductive biomarkers (and therefore their relationships to anthropogenic contaminants) can vary greatly with the season (Patiño, 1997). The results of the present study reflect the biological condition of the fish and its association with metal body-burdens at the time of the year that is perhaps most relevant ecologically: at the onset of the spawning period.

5. Conclusions

Just prior to their spawning season, male common carp from LMNRA could be grouped according to collection site on the basis of their metal composition or biological condition. Therefore, the general location in which common carp reside within LMNRA has a significant impact on their chemical and biological characteristics. Also, nearly one quarter of the variance in biological traits was explained by the variance in the overall metal composition of the fish. This level of shared variance between the variable sets is substantial and therefore relevant to our understanding of factors that influence fish health and reproductive condition. Similar observations were made in a companion study of spatiotemporal relationships between anthropogenic organochlorine contaminants and the biological condition of male common carp in LMNRA (R. Patiño et al., unpublished data). The results of the present study thus suggest that incorporation of metal bioaccumulation analyses into field studies of endocrine and reproductive dysfunction would enhance our understanding of impacts of anthropogenic activities in wild fish populations.

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