

Summary: *Histological examination, differential leukocyte counts, and bacterial culture was performed on adult carp collected from Las Vegas Bay and Overton Arm regions of Lake Mead in May, June, September, and November 1999. No significant difference or obvious impairment to general health was identified between the groups. While both populations showed histological signs of oxidative tissue damage and hemosiderosis (excess iron), Las Vegas Bay carp tended to have greater quantities of hepatocellular hemosiderin. It is likely that high levels of dietary iron promoted lipid peroxidation in these fish.*

Introduction:

In 1999, the California - Nevada Fish Health Center was a cooperator in an inter-agency study of endocrine disruption in adult common carp (*Cyprinus carpio*) from Lake Mead. This study was led by the US Geological Survey (Project Coordinator: Dr. Steve Goodbred, National Water Quality Assessment Program). A 1995 study of adult carp from the Las Vegas Wash and Bay region of Lake Mead reported that these fish showed evidence of endocrine disruption (Bevans et al. 1996). The current USGS study was designed to build on this earlier work by surveying adult carp in the Las Vegas Wash and Overton Arm region of Lake Mead for reproductive indicators and general health.

The Fish Health Center performed the following functions for the study: 1) histological evaluation of kidney, spleen, and hepatopancreas, 2) differential count of blood leukocytes and statistical evaluation of Lymphocyte : Granulocyte ratios, 3) culture and identification of bacteria isolated from blood, 4) participation in the development of field collection procedures and associated training (also assisted with May 1999 lake samples), and 5) provide histological fixative, blood smear supplies, and bacterial media for the project. Partial funding for supplies and lab technician time, totaling \$10,000, was provided to the Fish Health Center in February 2000 (USGS agreement 1434-HQPG-00-0013). *This report is limited to histopathological, differential leukocyte count, and bacterial isolation data generated by the Fish Health Center.* Readers should contact Dr. Goodbred for information on the other aspects of the study generated by the USGS (environmental, contaminant, endocrine, fertilization, reproductive organ morphology, etc.).

Methods

Sexually mature common carp (*Cyprinus carpio*) were captured in shallow water by use of an electro-shocking boat at 2 general locations in Lake Mead (Jim Pollard, Univ. of Nevada Las Vegas pers. comm.):

	<u>Latitude</u>	<u>Longitude</u>
Las Vegas Bay	N36° 07' 51"	W114° 51' 55"
Overton Arm	N36° 28' 13"	W114° 22' 56"

After capture, fish were held for up to several hours in an aerated live-box before blood and tissue collection. Collection dates and samples numbers are listed in Table 1.

Table 1. Collection dates and sample numbers from Las Vegas Bay (LV) or Overton Arm (OV) in 1999.

Date	Site	Histological Specimens	Blood smears	Bacterial Samples
10MAY	LV	31	21 **	30
11MAY	OV	30	28	30
07JUNE	LV	26	30	30
08JUNE	OV	26	30	30
13JULY	OV	5	5	5
14JULY	LV	Not done++	3	3
13SEPT	LV	22	22	22
14SEPT	OV	22	22	21
08NOV	LV	19	20	19
09NOV	OV	19	19	20

** Cytospin preparations rather than standard bloodsmear

++ Collected from Las Vegas Marina. High winds interfered with electro-fishing.

Blood – Stunned fish were measured for length and weight, and a blood sample collected from the caudal vessels with a heparinized syringe. The majority of the blood sample was centrifuged for plasma. A small quantity of blood within the syringe was reserved for bacterial culture and blood smears. The needle was wiped with 70% isopropyl alcohol, several drops of blood expressed, and 20 μ L inoculated onto a slant tube of Brain Heart Infusion agar for bacterial isolation and a microscope slide for bloodsmear preparation. Bacterial samples were maintained between 10 -25 °C and shipped with blue ice to the FHC within 3 days of collection. Standard microscopic and biochemical tests (such as API-20E) were used to identify isolated colonies to the genus level. Blood smears were air dried 10 minutes, fixed for 15 minutes in absolute methanol, and later stained with Leishman – Giemsa stains (Yasutake and Wales 1983). A differential leukocyte count was performed at 1000x magnification on the first 200 lymphocytes, thrombocytes, neutrophils, monocytes, and eosinophil / basophils observed on the smear. Leukocyte identification was based on Hibiya (1982) and Witten et al. (1998). Because there is poor morphological distinction between the low percentage of rounded thrombocytes (typically spindle-shape) and the more numerous lymphocytes, the combined number of lymphocytes + thrombocytes divided by the number of granulocytes (neutrophil and eosinophil / basophils) was used to derive a L{T}: G ratio. Higher granulocyte counts result in low L{T} : G ratio values and can indicate infection, tissue damage, or seasonal blood cell changes (Modra et al 1998). The use of combine eosinophil / basophil count was based on the observation of Moritomo et al. (1999) that carp blood basophils can become eosinophilic during typical staining procedures. Erythrocytes were examined for inclusions (viral infection) or karyorrhexis .

Histology - Two to six cm² sections of kidney, spleen, and hepatopancreas were dissected from the carp after blood collection and placed into 40 mL tubes of modified PREFER[™] fixative (Anatech Ltd, Battle Creek MI). Tissues were held for up to 20 days in this non-formalin fixative prior to standard paraffin block processing. The fixative (pH= 4.5) was composed of:

PREFER Concentrate (active ingredient is the aldehyde glyoxal)	300 mL
50% ethanol	1 L

Tissues were processed to 5 µm paraffin sections and replicate sections stained with hematoxylin and eosin, or Perls iron stain (Humason 1979). Iron stained sections rated for endogenous pigments were first viewed by brightfield microscopy to identify hemosiderin (blue) and melanin (brown-black). In Perls Prussian Blue stain, the ferrocyanide complexes with the ferric (Fe 3+) iron in hemosiderin but does not stain iron within hemoglobin (Kiernan 1981). The same section was then read for lipopigments under 360 nm excitation fluorescent light (Shimusaki et al 1989). The endogenous pigment (combined hemosiderin and lipopigments) of a given tissue was rated by a subjective 0,2,4, 6 severity scale and a mean group severity score calculated from the ratings (Reimschuessel et al. 1992).

0	None	
2	Mild	(1 – 10 % of specific tissue)
4	Moderate	(11 –49 % of specific tissue)
6	Extensive	(>50% of specific tissue)

Results

Bacterial isolates - No obvious difference in either prevalence of infection or isolate type was observed between the groups (Table 2). Based on the absence of clinical signs for bacterial infection (both gross observations and histological results) and the relatively few colonies per positive blood sample, it is likely that the bacterial isolates represent asymptomatic infections and were not indicators of impaired health. It is also likely that some isolates were due to external contamination of the blood sample. *Pseudomonas sp.* or *Aeromonas hydrophila* were the predominate bacteria isolated from both populations. All the bacteria isolated in the study can be found both within the intestinal tract of the fish and in the water.

Table 2. Bacterial isolates from blood samples (# positive / total samples).

	<u>Las Vegas Wash</u>	<u>Overton Arm</u>
<u>May</u>	0 / 30	0 / 30
<u>June</u>		
<i>Bacillus sp.</i>	2	3
<i>Staphylococcus sp.</i>	2	0
<i>Micrococcus sp.</i>	0	1
<i>Aeromonas / Pseudomonas</i>	5	13
No identification	2	0
	<hr/> 11 / 30	<hr/> 17 / 30
<u>July</u>	0 / 3 **	0 / 5
<u>September</u>		
<i>Bacillus sp.</i>	2	2
<i>Micrococcus sp.</i>	2	0
<i>Staphylococcus sp.</i>	0	2
<i>Vibrio cholerae</i> ++	1	0
<i>Aeromonas sp.</i>	7	6
	<hr/> 12 / 21	<hr/> 10 / 22
<u>November</u>		
<i>Bacillus sp.</i>	0	1
<i>Aeromonas sp.</i>	3	1
	<hr/> 3 / 20	<hr/> 2 / 19

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 ** All 3 carp captured at Las Vegas Bay Marina.

++ Presumptive based on API-20 database match.

Differential Leukocyte Counts - Cytospin preparations (Statspin, Norwood MA) were made for the May LV group. The longer processing time for cytospin did not mesh well with the other necropsy activities and standard blood smears were done for all subsequent groups. Any comparisons between the May LV and OV groups should be viewed with caution due to the different sample preparation methods. Except for September, there was a significant difference between the monthly sample groups (Mann-Whitney Rank sum test, $P < 0.002$). The mean LT:G ratio showed no consistent site trend as each sample group alternated its relationship with the other group on a monthly basis, however, the ratios tended to decrease over time (Fig. 1). There was considerable variation in LT:G values for each monthly group (coefficient of variation ranging from 48 – 140 %). An examination of outliers (those fish with a LT:G ratio < the monthly combine site 25 % quartile value) also showed the same alternating pattern (Fig 2.). Elevated granulocyte counts were generally the cause of the low LT:G ratios.

fig 1

The lymphocyte abundance tended to decrease over the May to November period in both groups while neutrophil counts remained steady (Table 3 and Figure 3). The November LV group showed the greatest decline in lymphocytes with corresponding low LT:G ratios. As evident by the increased microscopic search time, the absolute number of leukocytes per bloodsmear markedly declined in both November sample groups. Concurrently, there was a significant increase in the number of eosinophils in November. These granulocytes were relatively rare in May, June, and September samples. No significant correlation with endogenous pigment disposition in hepatopancreas, kidney, or spleen tissues and LT:G was seen in the sample groups (correlation coefficients ranged from $r^2 = 0.06$ to 0.4). Monocytes were extremely rare in the data set and no erythrocyte abnormalities were seen in either group.

Figure 2. Percentage of sample group with LT:G ratio \leq **monthly
combine site 25 % quartile value**

Table 3. Mean (\pm SEM) count of leukocytes from the Las Vegas Bay (LV) and Overton arm (OV) sample groups.

	<u>Lymphocytes</u>		<u>Neutrophils</u>		<u>Eosinophils</u>	
	LV	OV	LV	OV	LV	OV
May	161 (\pm 5)	139 (\pm 4)	8 (\pm 3)	11 (\pm 2)	0**	0**
June	128 (\pm 4)	123 (\pm 4)	11 (\pm 3)	7 (\pm 3)	0	0
Sept.	106 (\pm 14)	100 (\pm 5)	7 (\pm 1)	10 (\pm 2)	1	2 (\pm 1)
Nov.	66 (\pm 5)	102 (\pm 9)	13 (\pm 3)	7 (\pm 1)	36 (\pm 4)	17 (\pm 4)

** Mean value less than 1 indicates cell type rarely seen in sample group

Figure 3.

Histological data – Endogenous brown pigments, both lipopigments and hemosiderin, were seen in the majority of hepatopancreas, spleen, and kidney sections from both groups. The pigments were seen in 2 general distributions:

- 1) Within large vacuoles of mononuclear cells that formed discrete aggregates. These focal pigment deposits will be referred to as Pigment-laden Macrophage Aggregates (**PMA**) as shown in figure 4.
- 2) Cytoplasmic granules within hepatocytes referred to as **Hepatocellular**.

Low quantities of melanin were occasionally seen in kidney sections and were distinguished from other endogenous pigments by their uniform granular size, black coloration, and negative reaction to both 360 nm blue light and Perl's iron stain.

KIDNEY AND SPLEEN

No nephron (glomeruli and tubules) abnormalities were observed in any specimen. Cysts, within the interstitium, were seen in a low prevalence of kidneys from both sample groups (Table 4 and Figure 5). They were composed of a connective tissue capsule with an interior of granulomatous tissue and lipopigment. The origin of these cysts is not understood but may represent successful isolation and destruction of an irritant by the fish. Special bacterial stains, such as Brown & Brenn Gram and Acid fast, did not reveal bacteria nor were parasites associated with the cysts. Presumptive uric crystals (blue crystalline whorls) were occasionally seen in either the interstitium or degenerative tubules of fish from both groups (incidence LV 9%, OV 3%). Degenerative *Myxidium* – like spores were seen in the kidney interstitium of two OV fish.

Table 4. Pigment-laden macrophage aggregate scores and cyst prevalence in kidney samples.

	MAY		JUNE		JULY		SEPT.		NOV.	
	OV	LV	OV	LV	OV	LV	OV	LV	OV	LV
mean score	2.6	3.6	3.9	2.7	1.7	ND	2.7	2.4	2.3	3.2
median score	4	2	4	2	2	ND	2	2	2	2
prevalence cysts (%)	4/30 (13)	2 /31 (6)	1/16 (6)	1/26 (4)	0 / 5 (0)	ND	2/22 (9)	0/22 (0)	1/19 (5)	0/19 (0)

fig4

fig5

Lipopigment was the primary endogenous brown pigment (>90%) within the kidney PMAs while smaller quantities of hemosiderin (10 – 20 %) was only seen in kidneys from carp with extensive hepatocellular iron deposits (4- 6 severity ratings). Several sections demonstrated an early stage in lipopigment deposition with aggregates of lipid-filled “foamy” macrophages containing trace amounts of lipofuscin. It is unclear whether the macrophages were scavenging phospholipids from the immediate tissue or had migrated to the kidney. No consistent trend in prevalence or severity of kidney endogenous pigment was observed between the sites (Table 4).

The proportion of splenic PMA hemosiderin and lipopigment was highly variable among both collection groups. Hemosiderin was dominant in spleens of fish with hepatocellular iron deposits. No lesions or parasites were seen in the spleen sections.

HEPATOPANCREAS

Neoplastic changes were only observed in one LV fish captured in September (1/123 total LV fish ~ 0.8%) whose hepatopancreas showed a presumptive cholangioma (Fig. 6). In contrast, six of eighteen adult carp collected from the Colorado River below Lake Mead in a 1998 study (Foott and Harmon 1999) had proliferative bile duct foci (presumptive cholangioma). Both groups appeared to have adequate energy stores. While glycogen was commonly observed within the cytoplasm of hepatocytes throughout the year, defined fat vacuoles were only seen in the September (10% LV & 5% OV) and November (37% LV and 32% OV) groups. Glycogen vacuoles were identified by their irregular shape and PAS positive granular contents.

The lipopigment and hemosiderin composition of the PMAs varied considerable in hepatopancreas from both collection groups. As seen in the kidney, a few macrophages were observed in several hepatopancreas sections with “foamy” cytoplasm containing a yellow pigment (early lipofuscinogenesis). With the exception of the June OV fish, both the prevalence and mean severity score of hepatopancreas PMAs were similar for both collection groups (Fig. 7). There was some reduction in severity score over the collection period. The June OV collection group was also notable in the high prevalence (81 %) of hepatocellular hemosiderin unlike other OV collection groups (Fig 8). Except for June, the prevalence of severe hepatocellular hemosiderin rating (#6) was much greater in the LV fish (Fig. 8). Hepatocellular hemosiderin severity did not correlate with PMA scores in hepatopancreas, spleen, or kidney (linear regression correlation coefficients of June, September, and November groups ranged from $r^2 = 0.006$ to 0.15).). In hematoxylin and eosin stained sections, the hepatocellular hemosiderin appeared as diffuse brown granules in the cytoplasm and was shown by Perl’s stain to be entirely iron (Fig. 9).

fig6

fig7 & fig8

fig9

Discussion

The general health of the sampled carp, as evident by the normal tissue morphology and the lack of symptomatic bacterial or parasite infection, was judged to be good. Except for degenerative *Myxidium* spores seen in 2 OV kidneys, no other internal parasites were observed in the histological tissue sections. While various aquatic bacteria were isolated from 26 % of all blood samples, it is unlikely that they represented a health problem. This assertion is based on the absence of clinical signs for bacterial infection and the relatively few colonies per positive blood sample. It is also likely that some isolates were due to external contamination of the blood sample. Similar findings occurred in 1998 (Foott and Harmon 1999). Factors such as species tolerance to stressors and plentiful energy reserves (food availability) could modulate the overall health effects of contaminant exposure. Carp are considered to be “hardy” fish due to their tolerance of poor water quality such as high water temperatures and low oxygen levels (Panek 1987). It is assumed that food availability is not a limiting factor for Lake Mead carp.

Blood cell morphology appeared normal and the leukocyte composition did not deviate significantly from normal ranges in either sample population (Blaxhall 1972, Luskova 1997). Unlike the reports of altered L : G ratios in contaminant exposed fish (Modra et al. 1998, Svobodova et al. 1994), no consistent trend in Lymphocyte (thrombocyte) : Granulocyte ratios could be linked to either site or related to PMA severity. A seasonal shift towards higher granulocyte numbers was observed in both groups with a pronounced increase in circulating eosinophil / basophil numbers from the November groups. Modra et al. (1998) reports similar winter-time shifts in the lymphocyte : granulocyte ratios in carp. Cooler water temperatures reduce the relative effectiveness of lymphocytes in fish and increase their reliance on non-specific defenses such as phagocytic granulocytes (Secombes 1996).

The endogenous pigments, lipofuscin / ceroid {referred here collectively as lipopigments} and hemosiderin, were observed in the tissues of both the LV and OV populations. The pigments were most often found in large cytoplasmic vacuoles of mononuclear cells that formed discrete aggregates in the kidney, hepatopancreas, and spleen. Wolke (1992) reported that endogenous pigments are commonly found in macrophage aggregates of many species of fish. Matsche and Grizzle (1999) reported that lipofuscin was the most abundant pigment in macrophage aggregates of healthy catfish and that hemosiderin was most prevalent in fish with symptomatic *Aeromonas hydrophilia* infections.

Lipopigments, such as lipofuscin and ceroid, are polymerized residues of peroxidized lipids and proteins. These membrane bound pigments are usually within secondary lysosomes of the cell (Sokol and Brunk 1989). These authors consider the lipopigment ceroid, to be an early “immature” form of lipofuscin. The insoluble yellow-brown pigment, lipofuscin is often referred to as “aging pigment” (Cotran et al. 1989). Harmon (1989) states that isolated lipofuscin contains 10 – 25 % phospholipid, 1 – 2 % metal, and the remainder protein. This author differentiated the two related lipopigments by stating that ceroid is found in both mitotic and post-mitotic cells while lipofuscin is only found in post-mitotic cells.

Hemosiderin is a golden-brown pigment composed of aggregates of ferritin micelles and occurs when there is an excess of iron in tissue (Cotran et al 1989). Hemosiderosis in fish is usually associated with either hemolytic anemia or excess dietary / environmental iron intake (Thiyagarajah et al.1998). Low quantities of hemosiderin is typically concentrated in the reticuloendothelial cells of the spleen and kidney as they recycle iron from degraded erythrocytes.

Endogenous pigments in the hepatopancreas, spleen, and kidney were common to both populations and had been observed in carp collected from Lake Mohave in 1998 (Foott & Harmon 1999). This suggests a basin wide occurrence of environmental iron uptake and oxidative stress. Excessive intracellular iron can induce cellular injury and death due to lipid peroxidation of organelle membranes and mitochondrial dysfunction. Copper is also reported to initiate lipid peroxidation (Sokol et al. 1990). Marzabadi et al. (1988) reported that ferric iron induced markedly higher levels of lipofuscin in cultured rat cardiac cells exposed to 40 % oxygen conditions. They speculated that iron was stored within the lysosomes and induced lipid oxidation by the formation of free hydroxyl formation (HO) via an O₂-driven Fenton reaction :



Anti-oxidant Vitamin E added to the culture media reduced the rate of lipofuscinogenesis. Farag et al. (1994) reports that a 21 day exposure to sub-lethal levels of heavy metals from both waterborne and dietary sources produced lipid peroxidation in the kidneys of rainbow trout. Baker et al. (1997) demonstrated that elevated dietary iron intake resulted in lipid peroxidation of catfish liver (*Clarias gariepinus*) and associated depletion of the antioxidant alpha-tocopherol. High tissue levels of iron are not good indicators of its biological effects as lipid peroxidation proceeds as a self-perpetuating cascade after the initial free radical formation. Despite their elevated malondialdehyde (peroxidation product), the catfish appeared to be able to regulate their body pool of iron as muscle and liver iron concentrations were not significantly different between controls and experimental fish. Histopathological damage to the liver has also been reported in rainbow trout fed high levels of iron (Desjardins et al. 1987). It is possible that oxygen radicals produced during normal cell metabolism, in the presence of high intracellular iron (or other transition metals such as copper), could have caused the extensive lipopigment deposits observed in the carp.

It should not be assumed that adult carp “normally” demonstrate high levels of either lipopigment or hemosiderin in their hepatopancreas. In August 1999, adult were collected from several ponds within the Nevada Department of Wildlife’s Mason Valley Wildlife Management Area and examined histologically by the CA-NV Fish Health Center. Only 2 of the 29 hepatopancreas sections examined contained PMAs and none to the degree seen in the Lake Mead project. These fish were of similar size (290 – 760 mm) as the Lake Mead collection group carp. Pending scale analysis of the 1999 Lake Mead carp should provide age data, however, preliminary readings indicate the majority were 3 year old fish (S. Goodbred, USGS, pers. comm. Aug. 29, 2000). This finding would reduce an age bias in the PMA data. While macrophage aggregates are a normal feature of the kidney, spleen, and liver of many teleost, the extensive concentration of PMA in the hepatopancreas may serve as a biomarker for oxidative stress.

The variability in both the leukocyte values and endogenous pigment deposition seen in fish from both populations may be a function of a given fish’s residency at any one site. A sentinel cage study (2 – 6 mo.) using a *Catostomid* surrogate for the endangered Razorback suckers would remove the uncertainty in previous exposure history. The control population, held at a clean water hatchery, would provide better baseline data than feral animals captured from a reference site. The addition of biochemical indicators of oxidative stress (plasma Glutathione, super oxide dismutase, catalase, hepatopancreas peroxide content) would complement histological data on lipofuscin pigments. The relative numbers of apoptotic cells (programmed cell death) and cell proliferation (Proliferative Cell Nuclear Antigen) could be evaluated by immunocytochemistry in selected tissues. Iron concentration of the fish’s gill, intestinal tract and digesta would give some indication whether the high hemosiderin pigment concentrations was due to diet, RBC recycling, or waterborne sources. In the 1998 Lake Mead and Mohave study, aggregates of macrophages containing both hemosiderin and lipopigment were observed in histological sections of testes of fish from both lakes. The presence of this biomarker for oxidative damage should also be monitored in fish used for future gamete quality work.

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